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Investigating the role of mRNA capping enzyme in C-MYC function

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Investigating the role of mRNA capping enzyme in C-MYC function

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I would like to dedicate this thesis to my Gran: the strongest, wittiest, most caring and independent lady I have known. You will always inspire me and I miss you dearly.

Declarations

I hereby declare that the following thesis is based on the results of investigation conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the researchers or their publications. This dissertation has not in whole, or in part, been previously presented for a higher degree.

……

Olivia Lombardi

I certify that Olivia Lombardi has spent the equivalent of at least nine terms in research work in the School of Life Sciences, University of Dundee, and that she has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

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Professor Victoria Cowling

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Abbreviations

°C:	degrees celcius
40S:	small ribsosomal subunit
A:	adenosine
ACTB:	beta actin
ADP:	adenosine diphosphate
ALK:	anaplastic lymphoma kinase
APC:	adenomatous polyposis coli
APS:	ammonium persulfate
ar:	autoradiograph
ARE:	AU-rich element
ARK5:	5' adenosine monophosphate-activated protein kinase-related kinase 5
ASF/SF2:	alternative splicing factor/splicing factor 2
ATP:	adenosine triphosphate
ATPase:	adenosine triphosphatase
ATR:	ataxia telangiectasia and RAD3-related
BCL-2:	B-cell lymphoma 2
BET:	bromodomain and extra-terminal motif
bHLH-LZ:	basic helix-loop-helix leucine zipper
BIM:	BCL-2-like protein 11
BL:	basal-like
bp:	base pair
BRCA1:	breast cancer 1
BRD:	BET-bromodomain
BSA:	bovine serum albumin
BYSL:	bystin-like
C:	cytidine
CAK:	cyclin-dependent kinase activating kinase
CBC:	cap binding complex
CCND1:	cyclin D1
CDK:	cyclin-dependent kinase
cDNA:	complementary DNA
CE/RNGTT:	mRNA capping enzyme/RNA guanylyltransferase and 5' triphosphatase
C-FOS:	cellular FBJ osteosarcoma
ChIP:	chromatin immunoprecipitation
C-JUN:	cellular JUN
cl. PARP:	cleaved PARP
cm:	centimetre
CMTR:	cap methyltransferase
C-MYC:	cellular MYC
CO2:	carbon dioxide
co-IP:	co-immunoprecipitation
CRD:	coding region determinant
CRD-BP:	CRD-binding protein
CRISPR:	clustered regularly interspaced short palindromic repeats

CTD:	C-terminal domain
C-terminal:	carboxy terminal
CTR:	C-terminal repeats
D:	aspartic acid
DBD:	DNA-binding domain
DCP:	mRNA decapping protein
DMEM:	Dulbecco's modified eagle medium
DMSO:	dimethylsulphoxide
dMyc:	Drosophila MYC
DNA:	deoxyribonucleic acid
DNMT3A:	DNA methyltransferase 3 alpha
dox:	doxycycline
DRB:	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSIF:	DRB sensitivity-inducing factor
DSTT:	Division of Signal Transduction Therapy
DTT:	dithiothreitol
DXO:	decapping exoribonuclease
E-box:	enhancer box
ECM:	extracellular matrix
EDTA:	ethylenediaminetetraacetic acid
EGF:	epidermal growth factor
EHZ2:	enhancer of zeste 2
eIF:	eukaryotic initiation factor
EMT:	epithelial-mesenchymal transition
ER:	oestrogen receptor
ERK:	extracellular signal-related kinase
ESC:	embryonic stem cell
F12:	Ham's F12 nutrient mixture
FACS:	fluorescence-activated cell sorting
FBL:	fibrillarin
FBS:	fetal bovine serum
FCP1:	TFIIF-associating CTD phosphatase 1
g:	gram
G:	guanosine
G:	glycine
G:	gauge
G1 phase:	gap 1 phase
gag:	group-specific antigen
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GCN5:	general control of amino-acid synthesis 5
GFP:	green fluorescent protein
GMP:	guanosine monophosphate
GMP:	gemcitabine monophosphate
GO:	gene ontology
GRO:	global nuclear run-on
GSK-3:	glycogen synthase kinase 3
GST:	glutathione S-transferase
GTase:	guanylyltransferase

GTF:	general transcription factor
GTP:	guanosine triphosphate
H3:	histone 3
H3K27me3:	histone 3 trimethylated at lysine 27
H4:	histone 4
HAT:	histone acetyltransferase
HCF-1:	host cell factor 1
Hcy:	homocysteine
HDAC:	histone deacetylase
HeLa:	Henrietta Lacks
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2:	human epidermal growth factor receptor 2
HIF1α:	hypoxia-inducible factor 1α
HRP:	horseradish peroxidase
hTERT:	human telomerase reverse transcriptase
IC50:	half maximal inhibitory concentration
IGF:	insulin-like growth factor 1
IGF1R:	insulin-like growth factor 1 receptor
IMEC/M:	IMEC/C-MYC
IMEC/v:	IMEC/vec
IMEC:	immortalised mammary epithelial cell
IMPDH:	inosine-5′-monophosphate dehydrogenase
IP:	immunoprecipitation
IRES:	internal ribosome entry site
K:	lysine
kDa:	kilodalton
KPNA2:	karyopherin alpha 2
KRAS:	Kirsten rat sarcoma
KSR:	kinase suppressor of RAS
L-MYC:	lung carcinoma MYC
lncRNA:	long non-coding RNA
M:	molar
M:	mesenchymal
MAT1:	menage a trois 1
MAX:	MYC-associated factor X
MB:	MYC box
Me:	methyl group
MET:	mesenchymal-epithelial transition
mg:	milligram
MG132:	carbobenzoxy-Leu-Leu-leucinal
MGA:	MAX gene-associated
miRNA:	micro RNA
MIZ-1:	MYC-interacting zinc finger protein
ml:	millilitre
MMTV:	mouse mammary tumour virus
MNT:	MAX network transcriptional repressor
mol:	mole
mRNA:	messenger RNA

MTase:	methyltransferase
mTOR:	mammalian target of rapamycin
mTORC:	mammalian target of rapamycin complex
MXD:	MAX dimerization proteins
MYC:	myelocytomatosis
MZP:	mizoribine monophosphate
n/a:	not applicable
N:	first transcribed nucleotide
NAD+:	nicotinamide adenine dinucleotide
NC:	non-canonical
NCBP:	nuclear cap binding protein
NCK1:	non-catalytic region of tyrosine kinase 1
NCL:	nucleolin
NELF:	negative elongation factor
NF-κB:	nuclear factor-κB
ng:	nanogram
NGF:	nerve growth factor
NLS:	nuclear localisation signal
NMD:	nonsence mediated decay
NME1:	nucleoside diphosphate kinase A/non-metastatic cells 1
nmol:	nanomole
N-MYC:	neuroblastoma MYC
NPM:	nucleophosmin
NS:	non-specific
nt:	nucleotide
N-terminal:	amino terminal
ntp:	nucleotide triphosphate
NUDT:	nucleoside diphosphate-linked moiety X motif
OB:	oligonucleotide/oligosaccharide binding
ODC:	ornithine decarboxylase
p:	phosphate
PABP:	poly(A)-binding protein
PARN:	poly(A)-specific ribonuclease
PARP:	poly (ADP-ribose) polymerase
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
Pi:	inorganic phosphate
PI3K:	phosphoinositide 3-kinase
PIC:	preinitiation complex
poly(A):	polyadenosine
PPi:	inorganic pyrophosphate
PPS:	post-progression survival
PR:	progesterone receptor
pre-mRNA:	precursor mRNA
P-TEFb:	positive transcription elongation factor B
PTF:	pioneer transcription factor
PVDF:	polyvinylidene fluoride
qPCR:	quantitative PCR

qRT-PCR:	quantitative reverse transcriptase PCR
RAD3:	radiation-repair 3
RAM:	RNMT-activating miniprotein
RAS:	rat sarcoma
rDNA:	ribosomal DNA
RHO:	rhodopsin
RIPA:	radioimmunoprecipitation assay
RNA pol:	RNA polymerase
RNA:	ribonucleic acid
RNAi:	RNA interference
RNMT:	RNA guanine-7 methyltransferase
RPB1:	RNA polymerase II subunit A
RPM:	revolutions per minute
rRNA:	ribosomal RNA
RT:	reverse transcriptase
RT-PCR:	reverse transcriptase PCR
S phase:	synthesis phase
S/Ser:	serine
S2:	serine at position 2 of the RNA pol II CTD
S2p:	phosphorylated S2 residues
S5:	serine at position 5 of the RNA pol II CTD
S5p:	phosphorylated S5 residues
S7:	serine at position 7 of the RNA pol II CTD
S7p:	phosphorylated S7 residues
SAH:	S-adenosyl-l-homocysteine
SAHH:	S-adenosyl-l-homocysteine hydrolase
SAM:	S-adenosyl methionine
SCLC:	small cell lung cancer
SCP:	small CTD phosphatase
SDS:	sodium dodecyl sulfate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
Seq:	sequencing
shRNA:	short hairpin RNA
siRNA:	short interfering RNA
SL1:	selective factor 1
SMC1:	structural maintenance of chromosomes protein 1
snoRNA:	small nucleolar RNA
snRNA:	small nuclear RNA
SP1:	specificity protein 1
SPT:	suppressor of Ty
T/Thr:	threonine
T:	thymidine
TA:	tris acetate
TAD:	transcription activation/transactivation domain
TBS:	tris-buffered saline
TE:	tris EDTA
TEMED:	tetramethylethylenediamine

TF:	transcription factor
TGFβ:	transforming growth factor beta
TIP49:	TATA box-binding protein-interacting protein 49kDa
TIP60:	tat-interactive protein 60kDa
TNBC:	triple-negative breast cancer
TOP2A:	topoisomerase 2A
TPase:	triphosphatase
TREX:	transcription export complex
tRNA:	transfer RNA
TRRAP:	transformation-TAD-associated protein
TSC:	tuberous sclerosis
TSS:	transcription start site
U:	uridine
U:	unit
UBF:	upstream binding factor
UTR:	untranslated region
V:	volts
vec:	empty vector
VHL:	von Hippel-Lindau
v-Myc:	myelocytomatosis viral oncogene
WB/wb:	Western blot
WBL:	wobble
WDR5:	WD40-repeat protein 5
WNT:	wingless-type MMTV integration site family
WT:	wild-type
Y/Tyr:	tyrosine
ZO-2:	zona occludens 2
α:	alpha
β:	beta
γ:	gamma
Δ:	change in
µg:	microgram
µl:	microlitre
µm:	micrometre
µM:	micromolar

Abstract

C-MYC is a transcription factor and a potent driver of many human cancers. In addition to regulating transcription, C-MYC promotes formation of the mRNA cap which is important for transcript maturation and translation. However, the mechanistic details of C-MYC-dependent mRNA capping are not fully understood. Since anti-cancer strategies to directly target the C-MYC protein have had limited success, enzymatic co-factors or effectors of C-MYC present attractive alternatives for therapeutic intervention of C-MYC-driven cancers. mRNA capping enzyme (CE) initiates mRNA cap formation by catalysing the linkage of inverted guanosine via a triphosphate bridge to the first transcribed nucleotide. The involvement of CE in C-MYC-dependent mRNA capping and C-MYC function has not yet been explored. Therefore, I sought to determine whether C-MYC regulates CE, and whether CE is required for C-MYC function.

I found that C-MYC promotes CE recruitment to RNA polymerase II (RNA pol II) transcription complexes and to regions proximal to transcription start sites on chromatin. Consistently, C-MYC increases RNA pol II-associated CE activity. Interestingly, cells driven by C-MYC are highly dependent on CE for C-MYC-induced target gene expression and cell transformation, but only when C-MYC is overexpressed; C-MYC-independent cells or cells retaining normal control of C-MYC expression are insensitive to CE inhibition. C-MYC expression is also dependent on CE. Taken together, I present a bidirectional regulatory relationship between C-MYC and CE which is potentially therapeutically relevant. Studies here strongly suggest that inhibiting CE is an attractive

strategy to selectively target cancer cells which have acquired deregulated C-MYC.

Chapter 1 : Introduction

1.1 mRNA synthesis

1.1.1 Transcription in gene expression

Transcription is the first step in gene expression and is the process by which genetic information in nuclear DNA is utilised as a template to generate RNA molecules (Shandilya and Roberts, 2012). Messenger RNA molecules (mRNA), encoded by protein-coding genes, are processed during transcription and are then exported to the cytoplasm where they are translated (Hocine et al., 2010). This permits synthesis of functional protein products (Jackson et al., 2010). Transcription is a tightly controlled process and integrates extra- and intra- cellular stimuli to control appropriate cell behaviour (Barolo and Posakony, 2002; Medzhitov and Horng, 2009; Ng and Surani, 2011). Dysregulation of transcription leads to perturbed expression of genes, which can result in the initiation and progression of cancer and other diseases (Lee and Young, 2013). It is therefore of great interest to understand more about how transcription and associated events are regulated.

1.1.2 Promoter recognition and pre-initiation

The enzyme that synthesises mRNA from a DNA template is termed RNA polymerase II (RNA pol II) (Jonkers and Lis, 2015). RNA pol II also synthesises a number of non-coding RNAs including micro RNAs (miRNA), long non-coding RNAs (lncRNA) and small nuclear RNAs (snRNA) (Lee et al., 2004; Matera et al., 2007; Wang and Chang, 2011). On the other hand, RNA pol I synthesises 45S precursor ribosomal RNA (rRNA) and RNA pol III primarily

synthesises 5S rRNA and transfer RNA (tRNA) (White, 2008). mRNA synthesis is preceded by sequential recruitment of multi-subunit complexes called general transcription factors (GTFs), mediator and RNA pol II to specific DNA sequences found close to the transcription start site (TSS) known as core promoters (Allen and Taatjes, 2015; Grunberg and Hahn, 2013). Following RNA pol II association, GTFs TFIIE and TFIIH associate to complete the pre-initiation complex (PIC) (Sainsbury et al., 2015). Promoters are often sufficient for PIC assembly and subsequent basal levels of transcription. However, up- or down-regulation of PIC assembly (and of other events in transcription) can be conferred by the binding of additional transcription factors to regions of DNA called enhancers (Kadonaga, 2004; Levine et al., 2014; Nikolov and Burley, 1997). Transcription factors recruit additional co-activators such as chromatin remodelling enzymes to promote euchromatin formation. This aids PIC assembly, promoter opening and downstream transcriptional events (Voss and Hager, 2014). Enhancers can be close to transcription start sites or can regulate distal genes via DNA looping, and are bound by a plethora of different transcription factors in a sequence-, cell- and stimulus- dependent manner (Ernst et al., 2011; Levine et al., 2014; Spitz and Furlong, 2012).

1.1.3 Initiation and pausing

TFIIE is a heterodimer which bridges RNA pol II to TFIIH and stabilises/stimulates TFIIH (Maxon et al., 1994; Ohkuma and Roeder, 1994). TFIIH is composed of ten subunits including adenosine triphosphatases (ATPases), DNA helicases and the cyclin-dependent kinase activating kinase (CAK) complex (Compe and Egly, 2012). In the presence of nucleoside triphosphates, TFIIE and TFIIH recruitment stimulates DNA unwinding and

promoter opening together with RNA pol II phosphorylation and dissociation of several GTFs (while TFIIE and TFIIH remain associated). This allows transcription initiation to assume and RNA pol II begins to synthesise precursor-mRNA (pre-mRNA). The TFIIH CAK consists of cyclin dependent kinase 7 (CDK7), cyclin H and menage a trois 1 (MAT1), and mediates phosphorylation of RNA pol II on its C-terminal domain (CTD) concurrent with transcription initiation (Laybourn and Dahmus, 1990; Ohkuma and Roeder, 1994; Rossignol et al., 1997). This CTD structure lies within the largest subunit of RNA pol II (RPB1) and is unique throughout the polymerases; it is absent from RNA pol I and III. The CTD consists of tandem heptad repeats (52 in humans) with the canonical sequence $Y_1S_2P_3T_4S_5P_6S_7$, and is subject to several modifications throughout transcription which govern the timely association of regulatory factors and pre-mRNA processing machinery (Hsin and Manley, 2012; Zaborowska et al., 2016). CDK7 specifically phosphorylates the RNA pol II CTD at Ser5 (S5p). This is generally required for global transcription, although CDK7 recruitment can be stimulated to upregulate specific genes in certain conditions (Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Wang et al., 2015). RNA pol II S5 phosphorylation initiates 5’ N7-methylguanosine pre-mRNA capping (mRNA capping, Figure 1.1) (Ho and Shuman, 1999; Nilson et al., 2015; Trigon et al., 1998). The mRNA cap is important for transcript stability, further pre-mRNA processing, nuclear export and translation (discussed in section 1.2.4). RNA pol II typically synthesises 20-60 nts before TFIIE is exchanged for 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole sensitivity-inducing factor (DSIF, a heterodimer of suppressor of Ty 4 [SPT4] and SPT5) and negative elongation factor (NELF, a

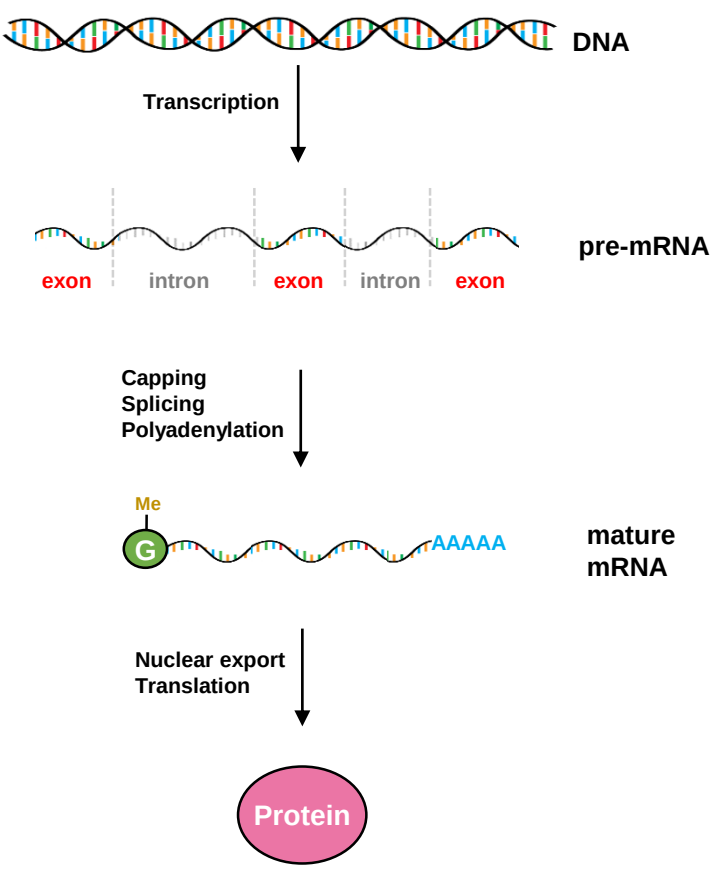

Figure 1.1: Pre-mRNA processing. During transcription, pre-mRNA molecules undergo modification to achieve maturation. Firstly, pre-mRNA molecules are capped with an N7-methylguanosine group at the 5' end. Introns are then removed and exons are conjugated together in a process termed splicing. Finally, transcripts are modified at the 3' end by addition of adenosines to form the poly(A) tail. Following processing, the mature mRNA is able to be exported into the cytoplasm and translated into the functional protein product.

four-subunit complex) (Core et al., 2008; Larochelle et al., 2012; Muse et al., 2007). DSIF and NELF cooperatively bind RNA pol II and nascent transcripts (Cheng and Price, 2008; Gebhardt et al., 2015; Missra and Gilmour, 2010). This occurs on around 30% of genes and induces a temporary arrest of transcription termed RNA pol II pausing (Figure 1.2) (Core et al., 2008; Wada et al., 1998a; Yamaguchi et al., 1999). Pausing permits pre-emptive assembly of the PIC such that transcription can be rapidly induced in response to signalling cues (Adelman and Lis, 2012; Bai et al., 2010; Wada et al., 1998b). It is also thought to serve as temporal window to allow the recruitment of mRNA capping machinery or for cap quality control (Mandal et al., 2004; Nechaev et al., 2010; Rasmussen and Lis, 1993).

1.1.4 Pause release and elongation

Pause release is stimulated and pre-mRNA synthesis is resumed upon recruitment of another kinase-containing complex – positive transcription elongation factor B (P-TEFb) – consisting of CDK9 and cyclin T (Marshall et al., 1996; Marshall and Price, 1995; Wada et al., 1998b). P-TEFb is activated by CDK7 (in the context of the TFIIH CAK module) which phosphorylates the CDK9 subunit, ensuring sequential occurrence of the different kinase activities (Larochelle et al., 2012). Although P-TEFb is globally required for transcription (Jonkers et al., 2014; Laitem et al., 2015), P-TEFb recruitment can be upregulated to specific genes by a range of signal-responsive transcription factors (Barboric et al., 2001; Eberhardy and Farnham, 2002; Jang et al., 2005; Yang et al., 2005). Mechanistically, the CDK9 subunit of P-TEFb phosphorylates DSIF and NELF, causing NELF dissociation from RNA pol II while DSIF remains associated, and this stimulates productive transcription

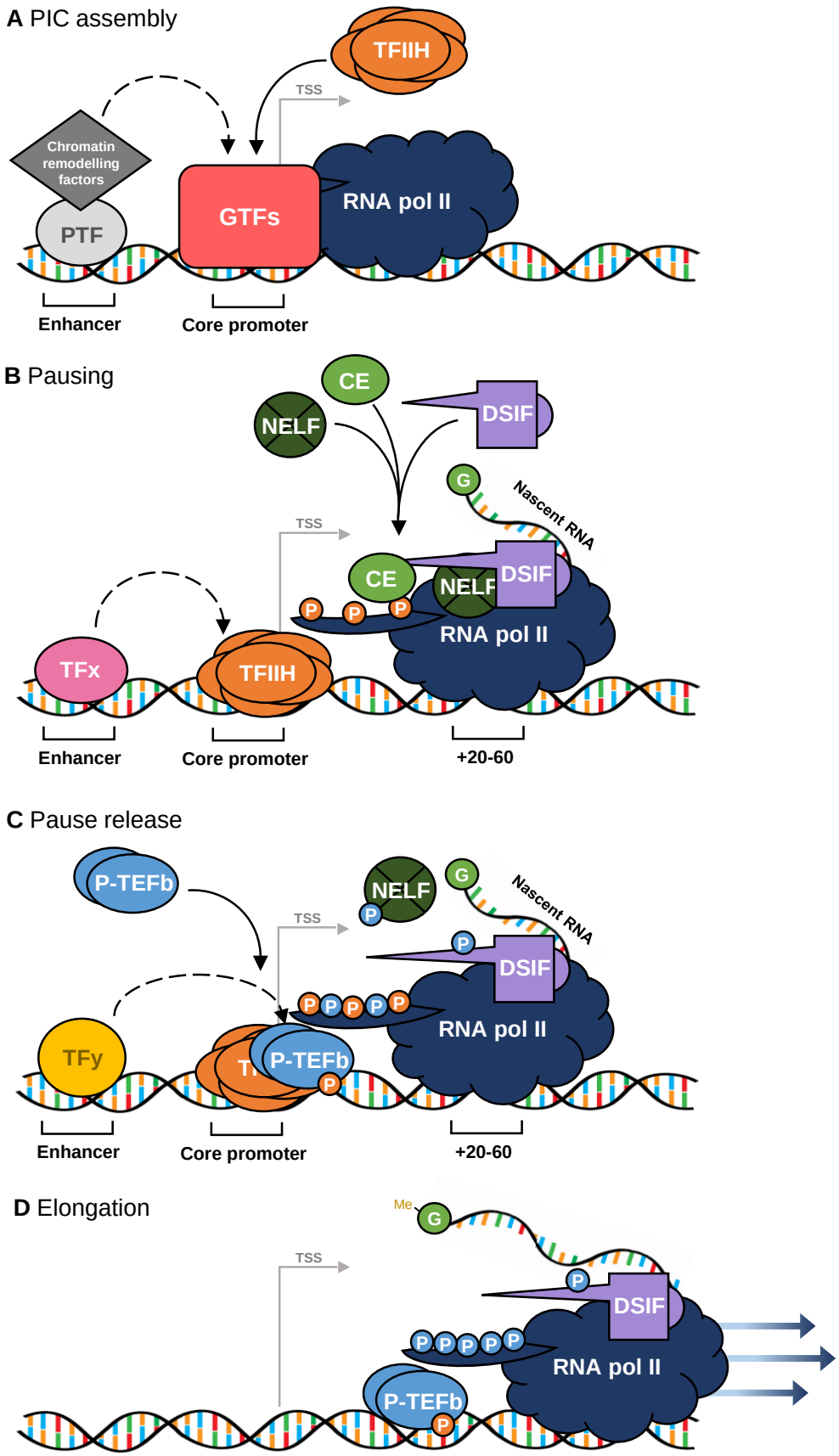

Figure 1.2: RNA pol II pausing. Legend overleaf.

Figure 1.2 continued: RNA pol II pausing.
A simplified model of RNA pol II pausing is depicted; described in more detail within the text. Solid arrows denote factor recruitment, whereas dashed arrows denote regulatory influence.

(A) TFIIH recruitment completes PIC assembly which initiates pre-mRNA synthesis. Pioneer transcription factors (PTF) can enhance PIC assembly by recruiting DNA/histone modifying enzymes.

(B) TFIIH (via its CDK7 catalytic subunit) phosphorylates the RNA pol II CTD at Ser5 residues (orange phosphate groups). After synthesis of 20-60nts, DSIF and NELF are recruited which induces RNA pol II pausing. CE is recruited to RNA pol II via phosphorylated Ser5 residues and caps nascent pre-mRNA. TFIIH recruitment can be enhanced by particular transcription factors (TFx).

(C) Transcription factors (TFy) can also enhance recruitment of P-TEFb. P-TEFb (CDK9 catalytic subunit) is activated by TFIIH and then phosphorylates the RNA pol II CTD at Ser2 residues (blue phosphate groups) in addition to NELF and DSIF. This causes dissociation of NELF and RNA pol II pause release.

(D) Upon pause release, transcription resumes and RNA pol II continues to synthesise pre-mRNA. As elongation progresses, phosphorylation levels of RNA pol II CTD Ser5 residues decline and that of Ser2 rise.

elongation (Andrulis et al., 2000; Fujinaga et al., 2004; Ivanov et al., 2000; Marshall et al., 1996; Renner et al., 2001; Wu et al., 2005). The SPT5 subunit of DSIF possesses C-terminal repeats (CTRs) analogous to the RNA pol II CTD (Figure 1.3), which when phosphorylated by CDK9 recruits transcription activators (Chen et al., 2009; Ivanov et al., 2000; Wier et al., 2013; Yamada et al., 2006). Therefore, CDK9 converts DSIF from a negative regulator to a positive regulator of transcription elongation. CDK9 also phosphorylates RNA pol II on its CTD at Ser2 residues (S2p), which mediates the recruitment of diverse factors such as those involved in splicing, histone modification and polyadenylation; influencing pre-mRNA processing, elongation and termination (Hsin and Manley, 2012; Ramanathan et al., 2001). Splicing is the process by which non-coding sequences (introns) are removed and coding sequences (exons) are ligated (Figure 1.1). This is mediated by the spliceosome – a large ribonucleoprotein complex – and occurs co-transcriptionally during elongation (Merkhofer et al., 2014). As RNA pol II CTD S2 residues become phosphorylated during transcription elongation, S5p residues are dephosphorylated by phosphatases termed SSU72 and small CTD phosphatases (SCPs) 1-3 (Xiang et al., 2010; Yeo et al., 2003).

1.1.5 Termination

As transcription approaches completion, amongst the last stretch of mRNA to be synthesised is a polyadenylation (poly(A)) signal (AAUAAA) followed by a G/U-rich sequence, which reduces RNA pol II processivity. Factors bind to the poly(A) signal and to S2p RNA pol II CTD which mediate sequential transcript cleavage, polyadenylation, and release of the mature mRNA from RNA pol II (Richard and Manley, 2009). The mRNA poly(A) tail is a

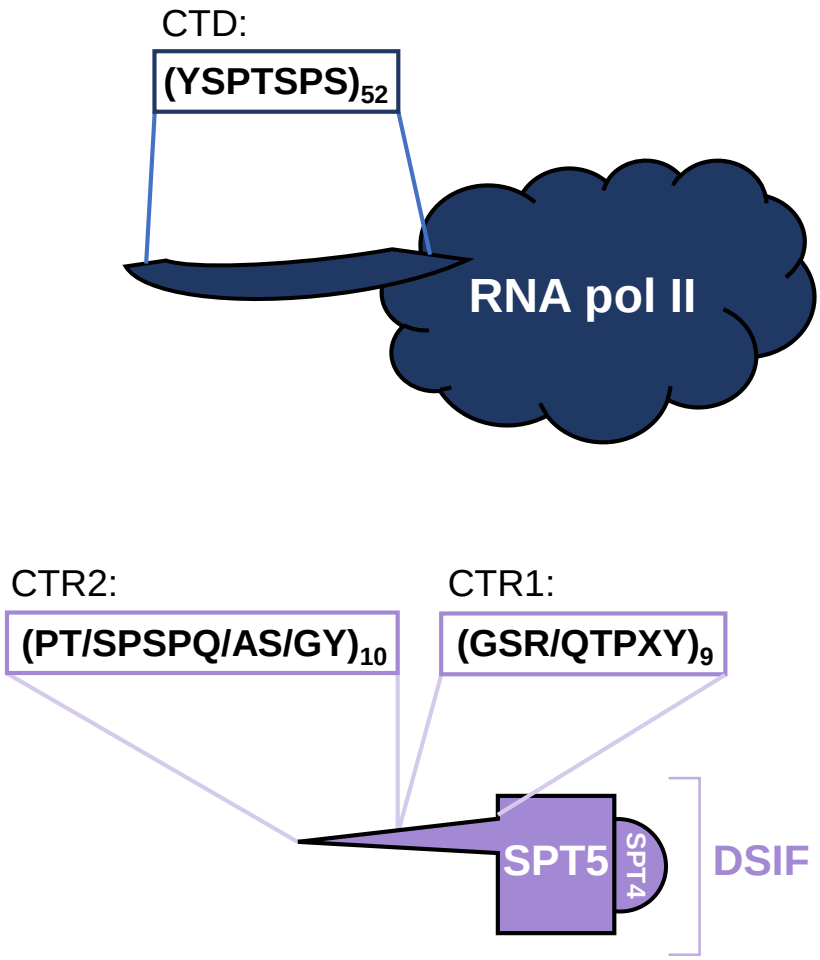

Figure 1.3: RNA pol II CTD and SPT5 CTRs. The SPT5 subunit of DSIF contains C-terminal repeats (CTRs) analogous to that in the RNA pol II CTD. Canonical sequences of the CTD and different CTR subdomains (CTR1 and CTR2) are shown. Of note, 21 CTD repeats adhere to the consensus sequence and the remaining 31 repeats can vary; mostly at position 7 but also at positions 2, 4 and 5. The RNA pol II CTD can be phosphorylated by both CDK7 and CDK9 on S5 and S2 residues, respectively. The T4 residues in the CTR1 domain of SPT5 are phosphorylated by CDK9 (Ivanov et al. 2000), whereas the CTR2 domain of SPT5 is phosphorylated by both CDK7 and CDK9 (Larochelle el al. 2006). Further studies are required to determine which residues in CTR2 are prefentially phosphorylated by these kinases. X represents a variable residue in CTR1.

stretch of typically 200-250 adenosines added to the 3’ of end of transcripts (Figure 1.1), which is not only important for termination, but also for mRNA stability by protecting transcripts from 3’-5’ exonucleases. Additionally, poly(A) tail-interacting proteins are important for promoting nuclear export and translation initiation (Goss and Kleiman, 2013). Before RNA pol II dissociation, S2p residues within the CTD are dephosphorylated by TFIIF-associating CTD phosphatase 1 (FCP1) in order for RNA pol II to be converted back to its unphosphorylated, pre-initiation-competent form (Chesnut et al., 1992; Cho et al., 1999; Ghosh et al., 2008). Although the precise mechanisms regarding RNA pol II disassembly remain incompletely understood, cleavage and termination factors induce RNA pol II pausing downstream of the poly(A) signal, which is thought to be key in this process (Proudfoot, 2016). This induces a conformational changes in the RNA pol II active site which likely destabilises the RNA pol II transcription complex (Zhang et al., 2015).

1.2 mRNA capping

1.2.1 The N7-methylguanosine mRNA cap structure and catalysis

N7-methylguanosine mRNA caps are found in eukaryotes and eukaryotic viruses, but not in bacteria or archaea. The eukaryotic N7-methylguanosine cap (also known as cap 0) is a guanosine moiety methylated on the N7 amine, conjugated to the 5' end of RNA molecules in a unique inverted conformation via a 5'-5' triphosphate linkage (Figure 1.4). mRNA molecules, as well as some non-coding RNAs including lncRNAs, primary miRNA transcripts and tRNAs, undergo N7-methylguanosine capping (Lee et al., 2004; Ohira and Suzuki, 2016; Quinn and Chang, 2016). The addition of this structure involves a multi-step process, which may be somewhat analogous between different RNA species but is best characterised in relation to mRNA. For the purpose of this thesis, N7-methylguanosine capping will be discussed in the context of mRNA and will be referred to as 'mRNA capping'. mRNA capping is the first pre-mRNA processing event on RNA pol II transcripts, which occurs co-transcriptionally as they reach 18-35 nt in length (Moteki and Price, 2002; Nechaev et al., 2010; Nilson et al., 2015; Rasmussen and Lis, 1993). Historically, mRNA capping was thought be a constitutive process which proceeded to completion on all mRNA molecules. On the contrary, several lines of evidence have demonstrated that mRNA capping can be regulated at multiple levels in various biologically relevant contexts (discussed in subchapter 1.3).

mRNA capping is the sum of three sequential enzymatic reactions – triphosphatase (TPase), guanylyltransferase (GTase) and methyltransferase (MTase) activities in that order – which in higher eukaryotes is mediated by two

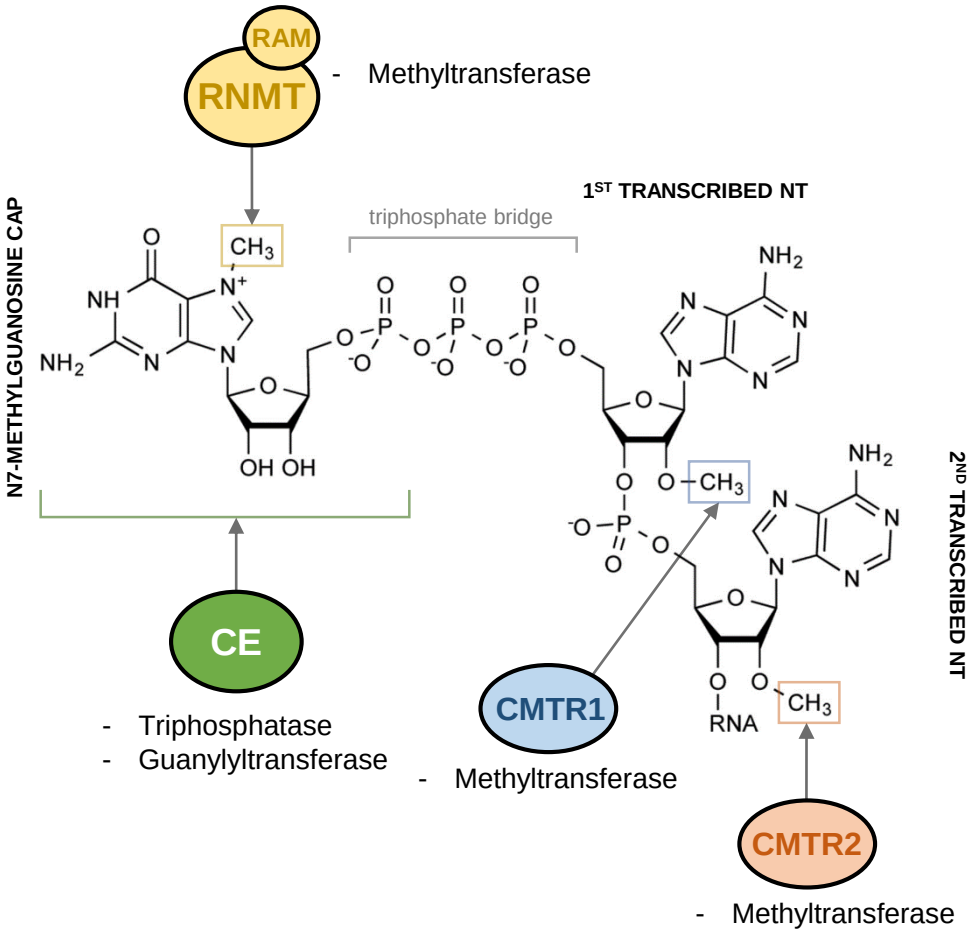

Figure 1.4: The mammalian mRNA cap and capping enzymes. The chemical structure of 'cap2' RNA is depicted together with the enzymes which catalyse mRNA capping. The frequency with which specific cap methylation events occur (i.e. the relative abundance of cap0, cap1 and cap2 mRNA) is still not clear. The catalytic activities of each enzyme are described. NT, nucleotide.

proteins (Figure 1.5). Firstly, the bifunctional protein capping enzyme/RNA GTase and 5'-phosphatase (CE/RNGTT) cleaves the terminal phosphate from the triphosphate group on the first transcribed nucleotide, yielding diphosphate RNA and inorganic phosphate (Pi). Secondly, CE acts via a two-step reversible ping-pong mechanism to catalyse cap guanylylation. CE hydrolyses guanosine triphosphate (GTP) to provide guanosine monophosphate (GMP) (with which it forms a covalent intermediate) and concurrently releases the inhibitory by-product inorganic pyrophosphate (PPi). CE then transfers GMP to the diphosphate group on the 5’ end of mRNA. Thirdly, RNA guanine-7 methyltransferase (RNMT) transfers a methyl group from S-adenosyl methionine (SAM) to the N7 position on the guanosine moiety to complete the mRNA cap (also termed cap0), meanwhile generating S-adenosyl-l-homocysteine (SAH) as an inhibitory by-product. Complete (N7-methylguanosine) and incomplete (guanosine) caps can be removed by decapping enzymes (discussed in section 1.3.4). It is worth noting that in lower eukaryotes (including yeast), the mRNA cap structure is conserved and cap catalysis is somewhat conserved, although the three activities contributing to cap formation are mediated by three distinct peptides.

Methylation of the first and second transcribed nucleotides on the 2’-O-ribose position (which occurs specifically in higher eukaryotes), is catalysed by cap methyltransferase 1 (CMTR1) and CMTR2, generating ‘cap1’ and ‘cap2’ structures (respectively) (Figure 1.4), both of which use SAM as a methyl donor (Belanger et al., 2010; Werner et al., 2011). Cap1 and cap2 structures are important for distinguishing ‘self’ RNA molecules from ‘non-self’ RNAs in the

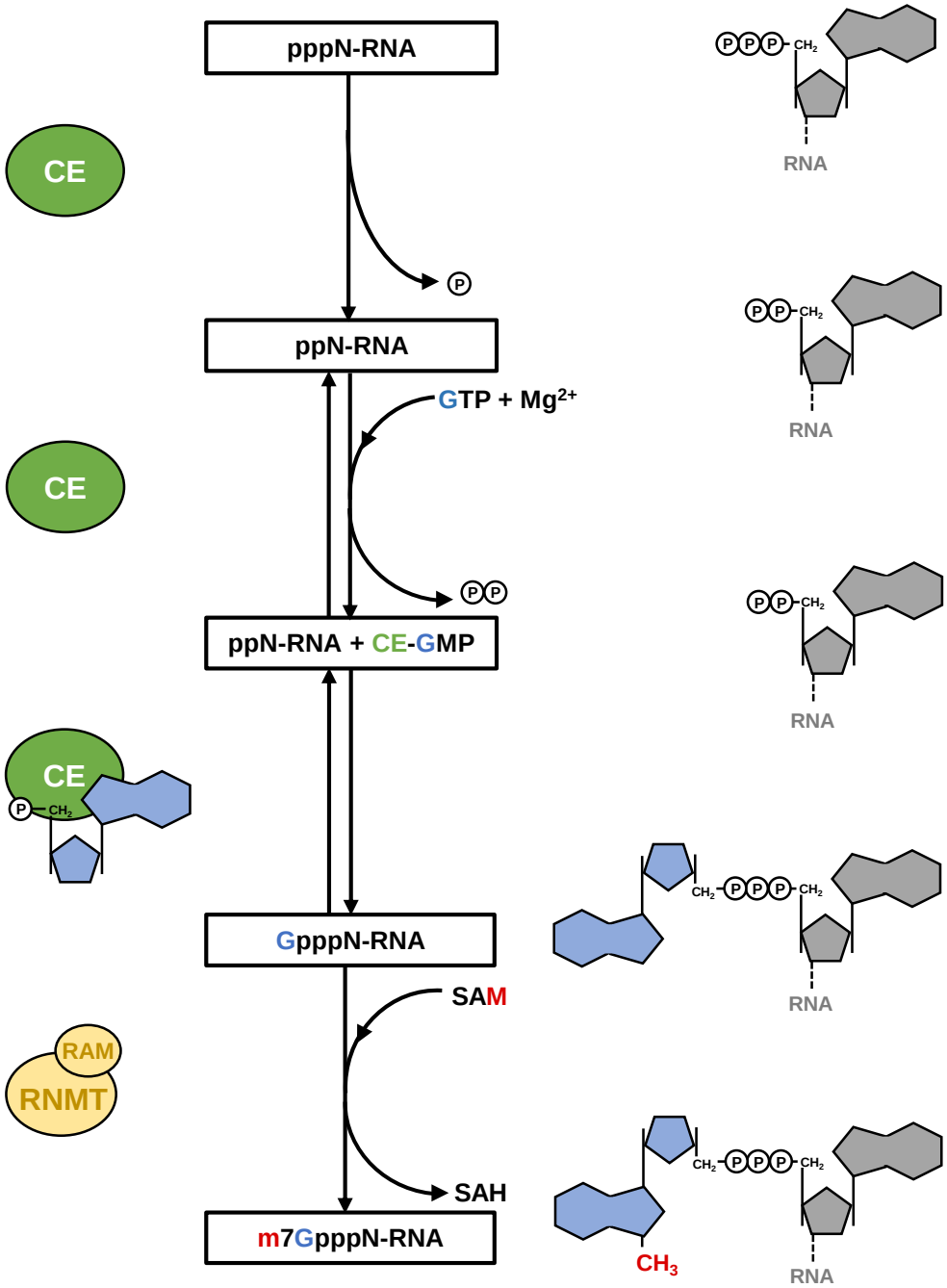

Figure 1.5: Mechanism of cap0 formation. The steps of mRNA capping (cap0 catalysis) by CE and RNMT are shown. CE cleaves the terminal phosphate from the triphosphate group on the first transcribed nucleotide, releasing Pi. In the presence of magnesium CE forms a covalent intermediate with GMP from GTP, releasing PPi. CE then transfers GMP to diphosphate RNA, yielding guanosine-capped RNA. In the presence of SAM, RNMT-RAM then methylates cap guanosine at the N7 position to complete the mRNA cap. As indicated, the two steps in the 'ping-pong' guanylyltransferase reaction are reversible. the guanosine cap nucleotide is depicted in blue and the first transcribed nucleotide is depicted in grey.

innate immune response (Abbas et al., 2017; Daffis et al., 2010; Schuberth-Wagner et al., 2015). Cap1 may also upregulate the translation of specific transcripts (Kuge et al., 1998; Kuge and Richter, 1995). Moreover, via an unidentified cytoplasmic methyltransferase, a reversible methylation event can occur on the first transcribed nucleotide at the N6 position of adenosine, which preferentially occurs on cap1 structures (Keith et al., 1978; Mauer et al., 2017). This promotes mRNA stability by preventing decapping (see section 1.3.4).

1.2.2 mRNA capping enzyme (CE/RNGTT)

As mentioned above, CE is a bifunctional enzyme with both TPase and GTase activities that act together to initiate mRNA capping by addition of the inverted guanosine group on the 5' end of nascent transcripts. CE belongs to a superfamily of covalent nucleotidyltransferases which include DNA/RNA ligases. The TPase domain resides at the N-terminal of CE and the GTase domain at the C-terminal. Within the GTase domain lies the active site (KxDG motif), in which the lysine contacts GMP, and also an oligonucleotide/ oligosaccharide binding (OB) domain which is involved in RNA-binding and protein-protein interactions. CE activity is coordinated with transcription (such that it caps nascent RNA as it emerges from RNA pol II) via interactions with S5p RNA pol II CTD and with 5' triphosphate RNA; CE cannot interact with unphosphorylated RNA pol II CTD (Ho and Shuman, 1999; Martinez-Rucobo et al., 2015). Consistent with this, dephosphorylation of the CTD by FCP1 depletes capping of nascent RNA in vitro by 70% (Mandal et al., 2004). Through a different binding site, CE also interacts with S2p RNA pol II CTD in vitro (Ho and Shuman, 1999). Moreover, yeast CE can interact with both S7p and S5p RNA pol II CTD, again via different CE binding sites (Bharati et al., 2016). However,

only S5p RNA pol II CTD peptides (and not S2p, S7p, nor other phospho-CTD peptides) were found to interact with CE in human cells (Pineda et al., 2015). Furthermore, when CE was co-crystallised with RNA pol II CTD peptides phosphorylated at both S2 and S5, CE only contacted S5p residues (Ghosh et al., 2011). In another study, inhibition of CDK7 (S5 kinase) but not CDK9 (S2 kinase) reduced co-transcriptional mRNA capping in vitro (Moteki and Price, 2002). Therefore, the predominant mode of contact between CE and the RNA pol II CTD is likely via S5p residues. In yeast, the lethal phenotype of Ser-Ala mutations at positions 5 (S5A) of the RNA pol II CTD can be rescued by tethering mammalian CE to S5A mutant RNA pol II (Schwer and Shuman, 2011), highlighting the conservation of CE activity and the importance of the CE-S5p RNA pol II interaction.

CE can also directly interact with the SPT5. Indeed, as previously mentioned SPT5 has a CTR with several Ser/Thr-Pro motifs analogous to that in the RNA pol II CTD (Figure 1.3) which are phosphorylated by CDK9 and CDK7 (Ivanov et al., 2000; Larochelle et al., 2006; Stachora et al., 1997). In yeast, CE interacts with the CTR domain of SPT5 (Pei and Shuman, 2002), although phosphorylation of yeast SPT5 CTR actually inhibits its interaction with the yeast capping enzymes (Doamekpor et al., 2014). Since the CTR and capping enzymes are substantially different to the mammalian homologues (Ghosh et al., 2011; Stachora et al., 1997), it is not clear if this mode of binding is conserved. Even though mammalian CE does not interact with yeast SPT5 CTR, overexpression of mammalian CE in yeast somewhat alleviates the slow growth phenotype of CTR deletion in SPT5 (Schneider et al., 2010). This indicates that the functional relationship between CE and SPT5 is conserved.

Interestingly, CE has roles in regulating RNA pol II transcription independent of its mRNA capping activity. The interaction of CE with SPT5 relieved DSIF and NELF-mediated RNA pol II pausing in vitro, and this was also true of a CE mutant devoid of its guanylyltransferase activity (Mandal et al., 2004). The variant of mouse CE (which is 95% similar to human CE) used in this study carries a substitution of the active site Lysine to Alanine (K294A), thereby CE guanylyltransferase activity is abolished while CE triphosphatase activity it retained (Yue et al., 1997). Consistent with CE recruitment regulating RNA pol II pausing/pause release, paused RNA pol II is phosphorylated at S5 (Cheng and Sharp, 2003) and CE and RNA pol II co-localise at putative pause sites on chromatin in cells (Glover-Cutter et al., 2008). Moreover, CDK7 inhibition caused a concurrent decrease in S5p RNA pol II phosphorylation, mRNA capping and RNA pol II pause efficiency in vitro (Nilson et al., 2015). Although pausing was not affected by inhibition of mRNA capping alone, the role of CE recruitment in pausing per se was not addressed (Nilson et al., 2015). Additionally, CE can promote the formation of co-transcriptional pre-mRNA:DNA hybrids termed R loops in vitro (Kaneko et al., 2007). Yet again, this was also true of the human K294A CE guanylyltransferase-dead variant (Kaneko et al., 2007), suggesting that CE recruitment rather than mRNA capping promotes R loop formation. R loops can promote transcriptional by protecting against DNA methylation at the TSS, amongst many other functions (Ginno et al., 2012; Santos-Pereira and Aguilera, 2015). In cells, the significance of CE in RNA pol II transcription is not yet clear.

1.2.3 mRNA guanine-7 methyltransferase (RNMT) and RNMT-activating miniprotein (RAM)

RNMT completes the final step in mRNA capping: methylation of the guanosine cap at the N7 position. N7 guanosine cap methylation is required for the interaction of cap-binding proteins with the cap (Izaurralde et al., 1994; Lee et al., 2016a; Lewis et al., 1996; Marcotrigiano et al., 1997). RNMT exists in a heterodimer with RNMT-activating miniprotein (RAM) which mediates RNA binding, structurally stabilises RNMT and enhances RNMT activity (Gonatopoulos-Pournatzis et al., 2011; Varshney et al., 2016). RNMT-RAM is recruited to transcription start sites in a CDK7-dependent manner (Aregger and Cowling, 2013; Posternak et al., 2017). However, an interaction between RNMT and RNA pol II is not readily observed (Aregger and Cowling, 2013; Pineda et al., 2015; Shatkin and Manley, 2000); only a weak ternary interaction between RNMT, CE and RNA pol II has been detected in vitro (Pillutla et al., 1998). Therefore the direct mechanism of RNMT-RAM recruitment is unknown.

Interestingly, RNMT promotes cell transformation; on its own or in co-operation with *C-MYC* and *RAS* oncogenes (Cowling, 2009). Additionally, RNMT regulates the expression of the cancer-associated genes cyclin D1 (*CCND1*) and *C-MYC* (Cowling, 2009; Dunn et al., 2016). Efforts are currently underway in the Cowling laboratory to understand the global impact of RNMT-RAM on gene expression in cancer cells.

1.2.4 Function of the mRNA cap and cap interactors

As previously alluded to, the mRNA cap is important for transcript stability, splicing, 3’ end processing, nuclear export and translation initiation

(Figure 1.6). Firstly, the mRNA cap itself stabilises transcripts during synthesis by preventing degradation by 5'-3' exonucleases (Furuichi et al., 1977; Murthy et al., 1991; Shimotohno et al., 1977). This is equally true of both the complete N7-methylguanosine cap and the incomplete guanosine cap. Other cap-dependent functions are conferred by cap-binding proteins, including the cap binding complex (CBC). CBC consists of the essential nuclear cap binding protein 1 (NCBP1) with NCBP2 (canonical CBC) or NCBP3 (alternative CBC) (Gebhardt et al., 2015; Gonatopoulos-Pournatzis and Cowling, 2014). NCBP2 or NCBP3 directly interacts with the mRNA cap, whereas NCBP1 stabilises NCBP2/3 and serves as an adaptor protein to recruit various factors. NCBP2 and NCBP3 have somewhat redundant functions under normal conditions, yet NCBP3 is important for cellular response to viral infection (Gebhardt et al., 2015). In vitro, CBC competes for cap binding with decapping enzymes (Jiao et al., 2013) and binds and inhibits poly(A)-specific ribonuclease (PARN) (Balatsos et al., 2006), which protects mRNA from both 5'-3' and 3'-5' exonuclease attack, respectively. CBC is an auxiliary factor for P-TEFb recruitment, and therefore promotes RNA pol II CTD S2 phosphorylation and efficient transcription elongation (Lenasi et al., 2011). CBC is resultantly important for S2p RNA pol II-dependent recruitment of alternative splicing factor/splicing factor 2 (ASF/SF2) and thus modulates alternative splicing (Lenasi et al., 2011). CBC also directly interacts with the U4/U6.U5 triple small nuclear ribonucleoprotein and thereby promotes co-transcriptional spliceosome assembly and splicing (Pabis et al., 2013). In addition, depletion of CBC causes defective 3' end pre-mRNA cleavage at the poly(A) site (Flaherty et al., 1997) although the mechanisms involved are not clear. Within the transcription export complex (TREX) the Aly/REF subunit associates with CBC, thus facilitating mRNA nuclear export

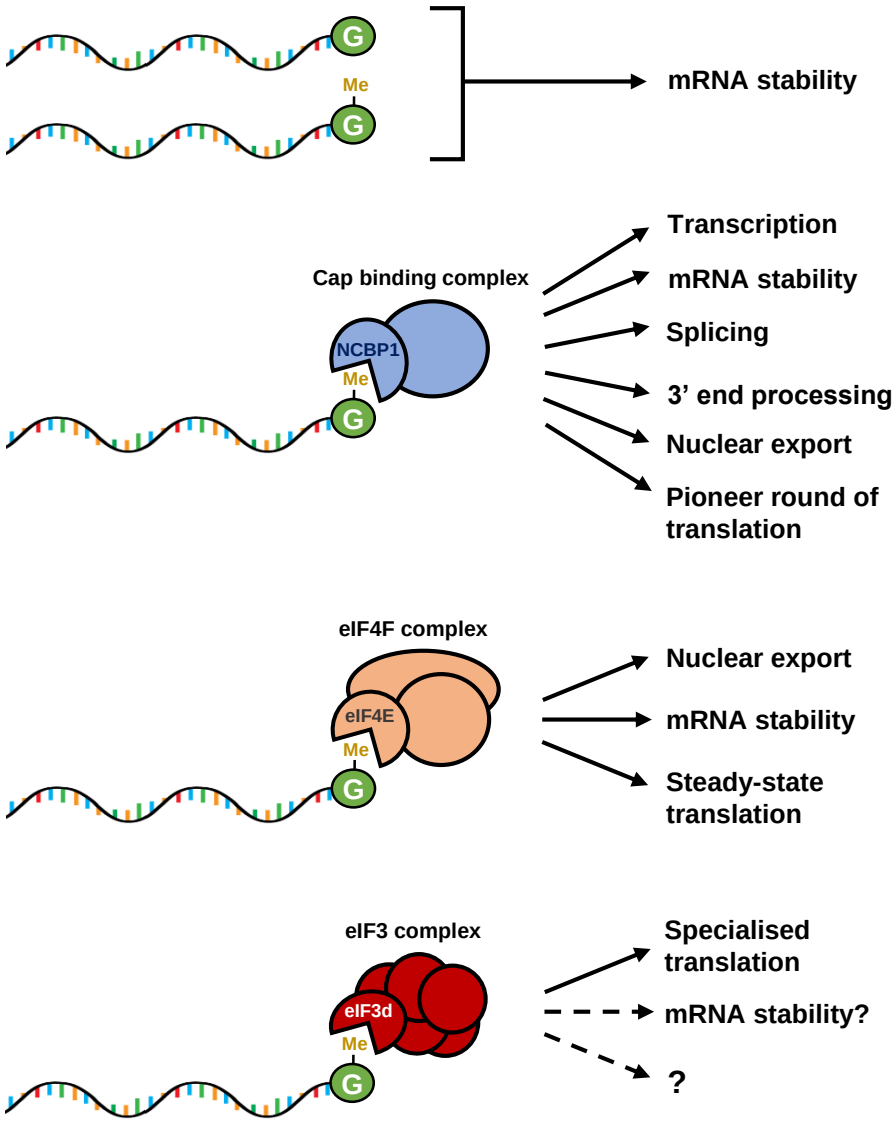

Figure 1.6: mRNA cap interactors and functions. The mRNA cap promotes many aspects of mRNA expression. 5' mRNA guanylylation is sufficient to confer resistance to 5'-3' exonucleic degradation. N7 methylation of guanosine is irreversible unlike 5' mRNA guanylylation, therefore this might indirectly provide additional protection in some systems. Moreover, N7 methylation of the cap guanosine is required for the interaction of cap binding proteins which protect from both 5'-3' and 3'-5' degradation. Cap binding proteins confer a host of functions, described in detail within the text. The novel eIF3d cap-binding protein was shown to promote translation of specific mRNAs, which is likely to be via other components of the eIF3 complex recruiting ribosomes. How eIF3d influences other features of capped mRNA such as their stability is not yet known.

(Cheng et al., 2006). Finally, CBC is able to associate with eukaryotic initiation factors (eIFs) and related proteins (in arrangements distinct from the classical translation initiation complex, eIF4F) and is thus able to mediate ribosome recruitment in the cytoplasm (Choe et al., 2012; Kim et al., 2009; McKendrick et al., 2001). Rather than promoting productive mRNA translation into functional protein products, CBC primarily functions in the pioneer round of translation which is important for mRNA quality control (Choe et al., 2012; Lejeune et al., 2004).

Another well-characterised cap-binding protein is the eIF4E translation initiation factor, which is a subunit of the eIF4F complex. The eIF4F complex typically orchestrates the majority of mRNA translation in the cell, and consists of an RNA helicase (eIF4A), and a large scaffold protein (eIF4G) in addition to the eIF4E cap-binding component. eIF4A disrupts secondary structures in the 5' untranslated region (UTR) of mRNAs whereas eIF4G makes supplementary contacts with mRNA and promotes mRNA looping in preparation for translation by bridging eIF4E and poly(A)-binding protein (PABP) (Kahvejian et al., 2005; Uchida et al., 2002; Yanagiya et al., 2009). Subsequently, eIF4G recruits the eIF3 complex which triggers ribosome assembly followed by translation of capped mRNA molecules (LeFebvre et al., 2006). eIF4E is also found in the nucleus, where it can directly mediate cap-dependent mRNA export (Cohen et al., 2001; Topisirovic et al., 2003a). Similar to the CBC, eIF4E competes with decapping enzymes for cap binding and thus stabilises transcripts (Grudzien et al., 2006). It is worth noting that the eIF4E is an oncoprotein which is regulated by oncogenic signalling pathways. Both eIF4E expression (for example via the oncogenic transcription factor C-MYC) and activity (via inhibitory

phosphorylation of eIF4E negative regulators) are subject to upregulation during cell transformation (Avdulov et al., 2004; Schmidt, 2004). The cap-binding capacity of eIF4E is essential for its functions and tumourigenic properties (Cohen et al., 2001; Ruggero et al., 2004; Topisirovic et al., 2003a; Topisirovic et al., 2003b).

An alternative cap-dependent translation pathway has recently been described. An eIF3 subunit (eIF3d) directly binds to the mRNA cap and mediates ribosome assembly and translation in an eIF4F-independent manner (Lee et al., 2016a). Interestingly, this upregulates the translation of a specific subset of mRNAs involved in cell proliferation such as *C-JUN* (Lee et al., 2016a; Lee et al., 2015). *C-JUN* possesses a stem-loop structure which prevents eIF4F-dependent translation and directs eIF3d recognition. Since eIF3d is structurally similar to a bifunctional decapping enzyme and exoribonuclease (decapping exoribonuclease, DXO) (Lee et al., 2016a) it is likely to promote mRNA stability via competition for cap binding.

1.3 Regulation of N7-methylguanosine mRNA capping

1.3.1 CE recruitment and activity

As previously discussed, mammalian CE has been shown to interact with S5p RNA pol II, SPT5 and S2p RNA pol II. Interestingly, in vitro, S5p RNA pol II CTD and SPT5 (but not unphosphorylated or S2p RNA pol II CTD) can stimulate CE guanylylation and mRNA capping 2-5-fold relative to free CE (Ghosh et al., 2011; Ho and Shuman, 1999; Mandal et al., 2004; Moteki and Price, 2002; Nilson et al., 2015; Wen and Shatkin, 1999). In concurrence, CDK7 inhibition, but not CDK9 inhibition, reduces mRNA capping (Moteki and Price, 2002; Posternak et al., 2017). Since S5p RNA pol II CTD contacts regions in the CE nucleotide binding domain, this might stabilise an open conformation of CE more conducive to guanylylation (Chu et al., 2011; Ghosh et al., 2011). Reciprocally, CE self-guanylylation stimulates its binding to S5p RNA pol II CTD; i.e. CE primed for mRNA capping has a greater affinity for RNA pol II (Ghosh et al., 2011). Signal-responsive transcription factors can upregulate S5 phosphorylation of RNA pol II (Aregger and Cowling, 2012; Cowling and Cole, 2007b), therefore it is possible that CE recruitment and activity might be modulated in response to stimuli, rather than being a constitutive process.

1.3.2 RNMT-RAM recruitment, activity and stability

The oncogenic transcription factor C-MYC has been shown to regulate mRNA cap methylation of C-MYC target gene transcripts and some other mRNAs, thereby enhancing mRNA translation and protein synthesis (Figure 1.7) (Cole and Cowling, 2009b; Cowling and Cole, 2007b; Fernandez-Sanchez et al., 2009; Posternak et al., 2017). C-MYC upregulates CDK7 expression and

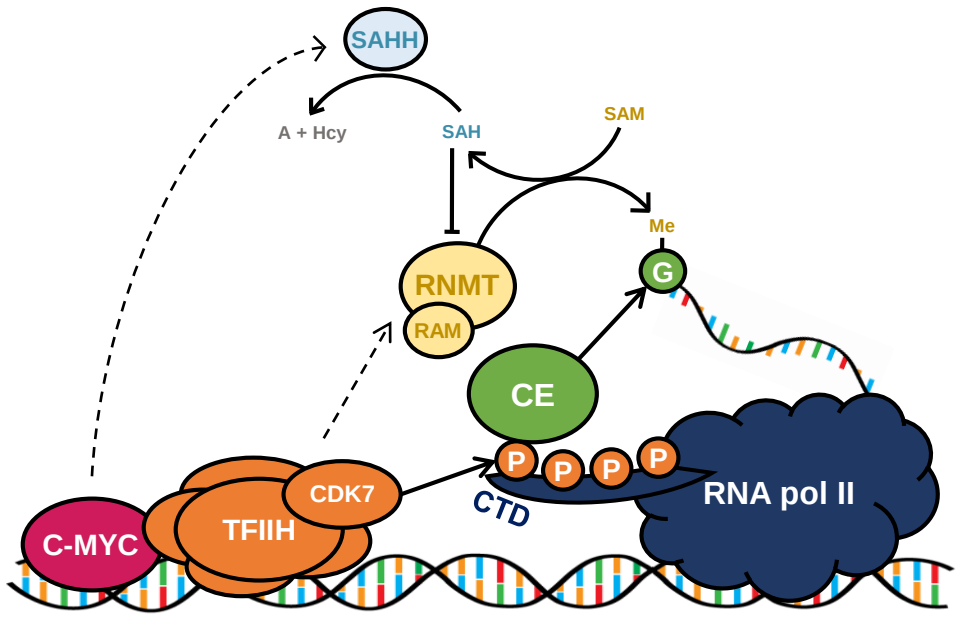

Figure 1.7: C-MYC regulates mRNA capping via RNMT and SAHH. C-MYC (and N-MYC) upregulate N7-methylguanosine mRNA capping. At least two mechanisms are thought to be involved. Firstly, C-MYC directly upregulates the *SAHH* gene, the protein product of which catalyses hydrolysis of SAH (the inhibitory by-product of cap methylation) into adenosine (A) and homocysteine (Hcy). Secondly, C-MYC recruits TFIIH containing CDK7 which phosphorylates RNA pol II, and this activity is required for recruitment of CE and RNMT. Solid arrows denote direct influence, whereas dashed arrow indicates indirect influence.

recruitment (Bouchard et al., 2004; Cowling and Cole, 2007b) thereby increasing RNMT-RAM recruitment to chromatin and mRNA cap methylation (Aregger and Cowling, 2013; Posternak et al., 2017). A C-MYC mutant lacking the ability to interact with CDK7 had a somewhat diminished potential to increase mRNA capping on some – but not all – C-MYC target genes (Cole and Cowling, 2009b), hinting that C-MYC might also regulate mRNA capping through additional mechanisms. Indeed, C-MYC was subsequently shown to directly upregulate the expression of SAH hydrolase (SAHH) which catalyses removal of SAH (the inhibitory by-product of the cap methylation by RNMT-RAM) (Fernandez-Sanchez et al., 2009). SAHH was shown to be required for C-MYC-dependent mRNA cap methylation and biological functions of C-MYC. Moreover, the SAHH inhibitor tubercidin (Radomski et al., 1999) reduced C-MYC-dependent proliferation, but not basal proliferation (Fernandez-Sanchez et al., 2009). It is worth noting that that the ability of C-MYC to increase mRNA cap methylation of target transcripts in these studies was determined by isolation of mRNA molecules using an antibody or recombinant eIF4E, which specifically recognise the complete N7-methylguanosine mRNA cap (and not the unmethylated guanosine mRNA cap) (Cole and Cowling, 2009b; Posternak et al., 2017). Therefore, only the final step in mRNA capping (i.e. cap methylation catalysed by RNMT-RAM) has been investigated, and it is not known if prior steps in mRNA capping are also rate-limiting in C-MYC function. Since the different steps in co-transcriptional mRNA capping occur with different kinetics (Moteki and Price, 2002), it is feasible that they could be differentially regulated.

RNMT-RAM is also regulated in a cell-cycle-dependent manner (Aregger and Cowling, 2012; Aregger et al., 2016; Cole and Cowling, 2009b). Similar to

C-MYC, the E2F1 transcription factor which is involved in cell cycle regulation upregulates mRNA capping (N7-methylguanosine mRNA cap addition) of its target gene transcripts, and this is dependent on its ability to stimulate RNA pol II phosphorylation (Aregger and Cowling, 2012; Cole and Cowling, 2009b). Moreover, RNMT is phosphorylated by CDK1 during the late S-phase and throughout G2/M-phases of the cell cycle, which coordinates RNMT-RAM activity with the peak of transcription at the beginning of the G1 phase (Aregger et al., 2016). RNMT phosphorylation both directly stimulates its catalytic activity and indirectly via relieving the inhibitory interaction of karyopherin alpha 2 (KPNA2) with RNMT (Aregger et al., 2016).

Furthermore, RNMT-RAM stability and activity are regulated during in vitro neural differentiation. High levels of RAM are required to maintain pluripotency in embryonic stem cells (ESCs), whereas during neural differentiation extracellular signal-related kinase (ERK) mediates phospho-dependent ubiquitination and degradation of RAM (Grasso et al., 2016). This is associated with downregulation of pluripotency genes and upregulation of neural genes.

1.3.3 CMTR1 expression

As previously mentioned, CMTR1 marks self-RNAs and therefore distinguishes them from viral RNAs during the innate immune response (Daffis et al., 2010). Consistently, expression of *CMTR1* is stimulated by interferon in response to viral infection (Geiss et al., 2003; Guerra et al., 2003; Haline-Vaz et al., 2008; Su et al., 2002). Moreover, since SAM is the methyl donor for CMTR1

and CMTR2 as well as RNMT (Haline-Vaz et al., 2008), their activity also has the potential to be regulated by C-MYC.

1.3.4 mRNA decapping and re-capping

An emerging concept is that mRNA decapping is a regulated process. Decapping is the process by which the cap is removed and precedes transcript degradation by exonucleases, which recognise 5'-monophosphate RNA (the RNA product of most decapping enzymes). At least four mRNA decapping enzymes are functional in mammalian cells: decapping mRNA 2 (DCP2), nucleoside diphosphate-linked moiety X motif 16 (NUDT16), NUDT3 and DXO. DCP2, NUDT16 and NUDT3 recognise the complete N7-methylguanosine cap or incomplete guanosine cap, and cleave between the α- and β- phosphates of the triphosphate group (Figure 1.4) to generate N7-methyl-GDP and 5′-monophosphate RNA (Song et al., 2013; Song et al., 2010; Wang et al., 2002). NUDT3 can also cleave between the β- and γ- phosphates of the cap, generating N7-methyl-GMP and 5′-diphosphate RNA (Song et al., 2013). The function of the latter is not clear, but it may be a substrate for re-capping (see below). DXO is a multifunctional enzyme which preferentially removes the incomplete mRNA cap (the unmethylated guanosine cap) in cells and removes the entire structure in conjunction with the first transcribed nucleotide (N), generating GTP-N and 5' monophosphate RNA (Jiao et al., 2013). It also has two activities towards uncapped mRNA: it cleaves between the α- and β-phosphates of the triphosphate group on the 5' end of RNA (generating N7-methyl-GDP and 5′-monophosphate RNA) and possesses intrinsic 5'-3' exonuclease activity (Jiao et al., 2013). Additionally, DXO was shown to act on a recently identified 5' nicotinamide adenine dinucleotide (NAD^+) cap structure

present on mRNAs, snRNAs and small nucleolar RNAs (snoRNA) in human cells (Jiao et al., 2017). Although for the purpose of this thesis it will not be discussed at length, NAD^+ caps – in contrast to m7G caps – destabilise RNA molecules and do not influence translation. NAD^+ caps actively promote DXO recruitment to mediate degradation of the associated RNA (Jiao et al., 2017).

DCP2 is the most studied decapping enzyme. It binds and decaps a subset of RNAs, which is achieved through recognition of specific RNA sequences containing a stem-loop structure within the first 10 transcribed nucleotides (Li et al., 2008; Piccirillo et al., 2003). DCP2 can bind mRNAs co-transcriptionally in the nucleus but is thought to act primarily in the cytoplasm, and has a regulatory interacting partner DCP1 with which it forms a heterodimer (Brannan et al., 2012; Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002). Various different factors regulate the DCP2-DCP1 interaction, DCP1 recruitment, DCP2 catalytic activity, DCP2 expression and DCP2 stability (Castellanos-Rubio et al., 2016; Erickson et al., 2015; Fenger-Gron et al., 2005; Jia et al., 2012). DCP2-mediated decapping is involved in both nonsense-mediated decay (NMD) and AU-rich element (ARE) mRNA decay pathways, and specific DCP2-interacting partners influence which of these pathways takes preference (Erickson et al., 2015; Fenger-Gron et al., 2005; Song and Kiledjian, 2007). DCP2 can be phosphorylated by mammalian target of rapamycin (mTOR), triggering assembly of a decapping complex which promotes the degradation of autophagy-related mRNAs (Hu et al., 2015). Furthermore, nuclear factor-κB (NF-κB) can transcriptionally upregulate *DCP2*, which causes downregulation of a long-non-coding RNA associated with celiac disease (Castellanos-Rubio et al., 2016). DCP2 is also negatively regulated, for example

PABP was shown to interact with the mRNA cap and thereby competes for DCP2 cap-binding in vitro (Khanna and Kiledjian, 2004) and reversible methylation of the first transcribed nucleotide at the N6 position of adenosine confers resistance to DCP2 (Mauer et al., 2017).

NUDT16 and NUDT3 are cytoplasmic decapping enzymes which also regulate a small subset of mRNAs (Grudzien-Nogalska et al., 2016; Song et al., 2010) although how they achieve specificity is not yet clear. Interestingly, through negatively regulating specific mRNAs, NUDT3 suppresses breast cancer cell migration (Grudzien-Nogalska et al., 2016). Although little is known about the biological role of DXO to date, it acts preferentially on nascent (unspliced) pre-mRNAs rather than mature (spliced) mRNAs and thus likely acts co-transcriptionally in the nucleus (Jiao et al., 2013).

In a further layer of complexity, decapping may not always result in transcript degradation, and some transcripts were shown to be re-capped in the cytoplasm. These mRNAs are enriched for those involved in the cell cycle, nucleotide binding and RNA/protein localisation (Mukherjee et al., 2012). As previously mentioned, decapping usually generates 5’ monophosphate RNA which is not a substrate for capping. However, in the cytoplasm CE interacts with an adaptor protein non-catalytic region of tyrosine kinase 1 (NCK1) which bridges the interaction of an unknown 5’ kinase to yield a 5’-diphosphate RNA substrate for CE (Mukherjee et al., 2014). Although the guanosine cap is methylated during re-capping, RNMT is not detected in the cytoplasmic capping complex and therefore the cap methylation mechanism is not known (Otsuka et al., 2009). Re-capping of mRNAs promotes their stability and translation (Mukherjee et al., 2012). Since NCK1 has roles in signal transduction (Castello

et al., 2013; Yiemwattana et al., 2012), it is possible that assembly of this complex is regulated. However, since CE is primarily nuclear, the biological significance of re-capping is not yet clear.

In conclusion, recent evidence highlights that mRNA capping and cap homeostasis are regulated at multiple levels. The relative contribution of the above mechanisms to gene expression is not known, and their biological relevance is not fully understood.

1.4 The *MYC* oncogenes

1.4.1 *C-MYC* discovery

The myelocytomatosis viral oncogene (*v-Myc*) was originally isolated from a chicken retrovirus (MC29) which caused solid, promyelocytic tumours (Ivanov et al., 1964). *v-Myc* in this viral strain was found fused to the viral *gag* (group-specific antigen) gene (Duesberg et al., 1977; Hu et al., 1979) and subsequently a homologous gene in vertebrates was identified (designated cellular *Myc*, or *C-MYC*) (Roussel et al., 1979; Sheiness and Bishop, 1979). *v-Myc* was then identified in other oncogenic retroviruses (Graf and Beug, 1978), and insertion of other retroviral promoters from viruses other than MC29 greatly stimulated *C-MYC* expression and caused transformation (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1981) adding to the notion that *v-Myc* is a retroviral oncogene and *C-MYC* its cellular homologue. This, together with the cloning of *C-MYC* in 1982 (Vennstrom et al., 1982) stimulated the wealth of further studies on the cellular oncogene.

1.4.2 Structure and function of MYC proteins

The *MYC* family of genes encode nuclear transcription factors which are conserved amongst metazoans and in premetazoans (Hartl et al., 2010; Simionato et al., 2007; Young et al., 2011). The normal function of MYC proteins is to control cell proliferation in response to extracellular growth cues via numerous signalling pathways. In humans there are three main family members designated *C-MYC, N-MYC and L-MYC*. They are of the basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor family, and form heterodimers with another bHLH-LZ transcription factor called MYC-associated

factor X (MAX) to regulate gene expression. MYC-MAX not only binds to enhancer boxes (E-boxes) with specific sequences (Figure 1.8) proximal to transcription initiation sites, but also to enhancers and other genes without clear sequence specificity in particular circumstances (Figure 1.9) (Blackwood and Eisenman, 1991; Fernandez et al., 2003; Kress et al., 2015). All MYC proteins have an N-terminal transcription activation domain (TAD), which is able to recruit various factors to drive or repress the expression of target genes (discussed in sections 1.4.6 and 1.4.7, respectively), and a C-terminal DNA-binding domain (DBD) (Figure 1.10). In contrast, MAX only has a DBD and does not possess a TAD (Kato et al., 1992). The LZ helix of the MYC DBD interacts with that of MAX, and the two basic helices bind the major groove of DNA (Nair and Burley, 2003).

All *MYC* paralogues are bona fide oncogenes. When constitutively activated, they increase cell growth/proliferation and cause cell transformation (Birrer et al., 1988; Lee et al., 1985; Yancopoulos et al., 1985) and they have roles in both tumour initiation and progression (Gabay et al., 2014). MYC proteins are structurally and functionally similar, but differ in expression patterns and potencies. *C-MYC* is widely expressed during development and throughout adult tissues, often highly in proliferating tissues (Downs et al., 1989; Schmid et al., 1989; Zimmerman et al., 1986) including in stem cell compartments where it maintains cell pluripotency and self-renewal (Cartwright et al., 2005; Takahashi and Yamanaka, 2006; Varlakhanova et al., 2010). Conversely, C-MYC also promotes apoptosis. Different levels of C-MYC activity tend to govern whether a cell proliferates or undergoes cell death (specific examples discussed in section 1.4.10) as an intrinsic tumour-suppressor mechanism (Murphy et al., 2008).

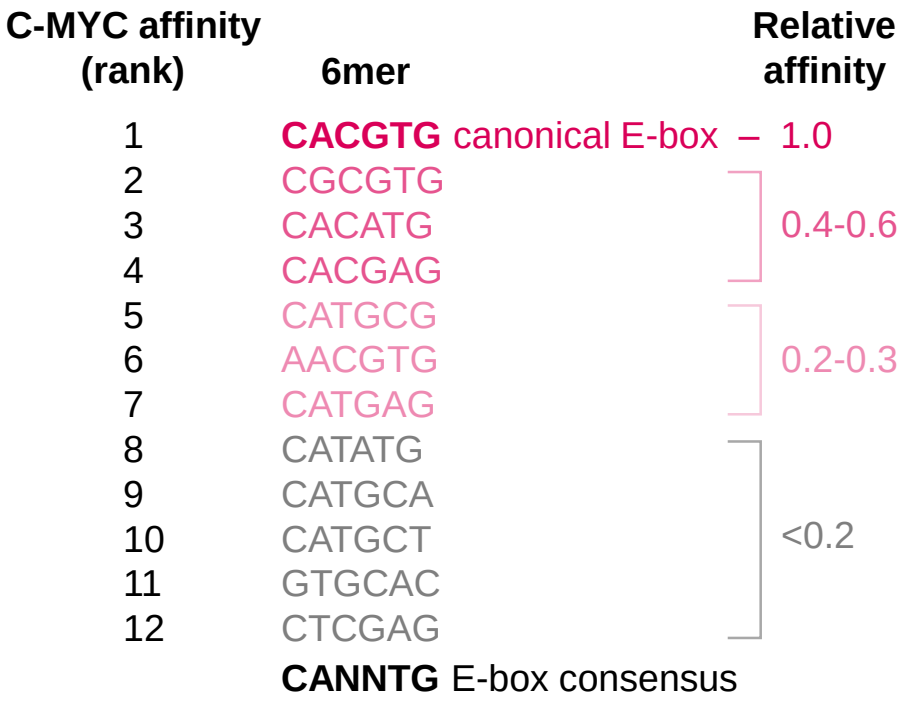

Figure 1.8: C-MYC sequence specificity. Relative affinities are shown for C-MYC-MAX dimers binding to the indicated 6mers in vitro. Shown are the top twelve 6mers in terms of C-MYC affinity of all possible 6mers, which includes the canonical E-box, non-canonical E-boxes and variants, and other 6mers. Notably, sequence specificity does not always predict C-MYC genome binding and gene regulation in cells; chromatin, RNA pol II status and C-MYC expression level also play prominent roles. Affinity data from Guo et al. 2014. N denotes any nucleotide.

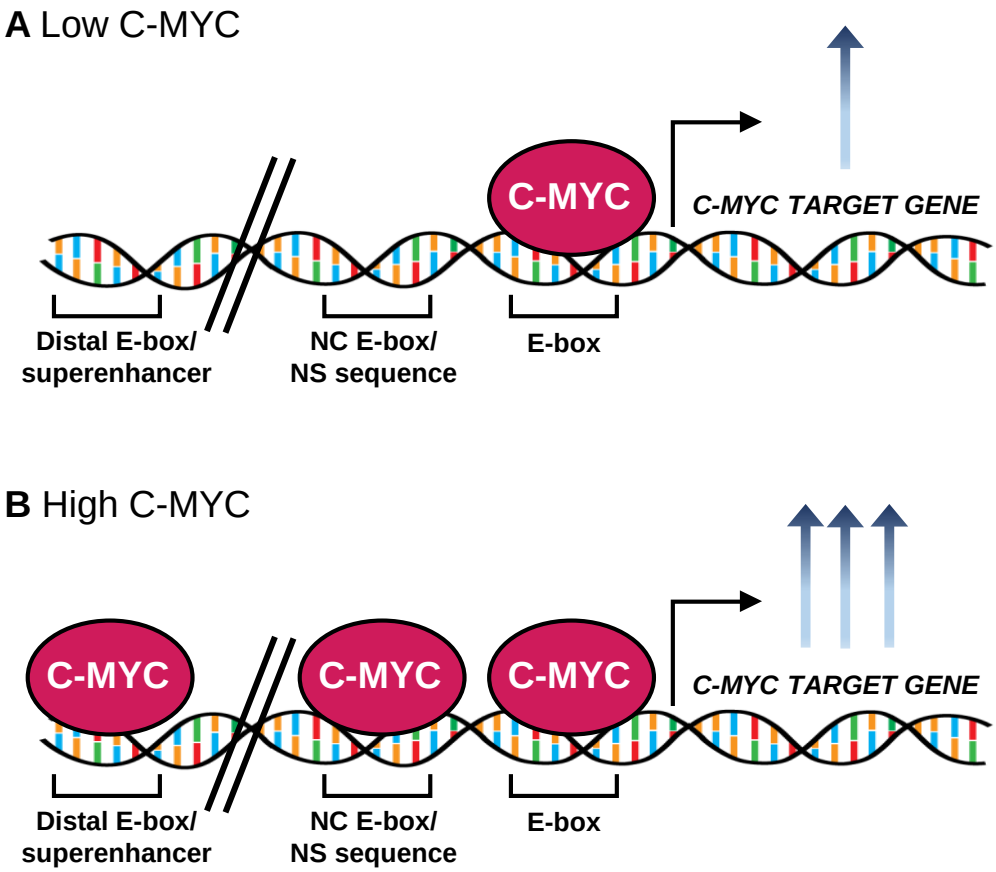

Figure 1.9: Dosage model of C-MYC recruitment. (A) In the presence of low C-MYC levels, such as in most somatic cells, C-MYC binds primarily to promoter proximal canonical E-boxes, promoting basal transcription. (B) In the presence of high C-MYC levels, such as in cancer cells, C-MYC also binds non-canonical (NC) E-boxes and non-specific (NS) DNA sequences in addition to distal regulatory elements and super-enhancers. This can cause elevated expression of C-MYC target genes and non-classical target genes. C-MYC recruitment is also influenced by chromatin context, C-MYC interacting partners and RNA pol II status.

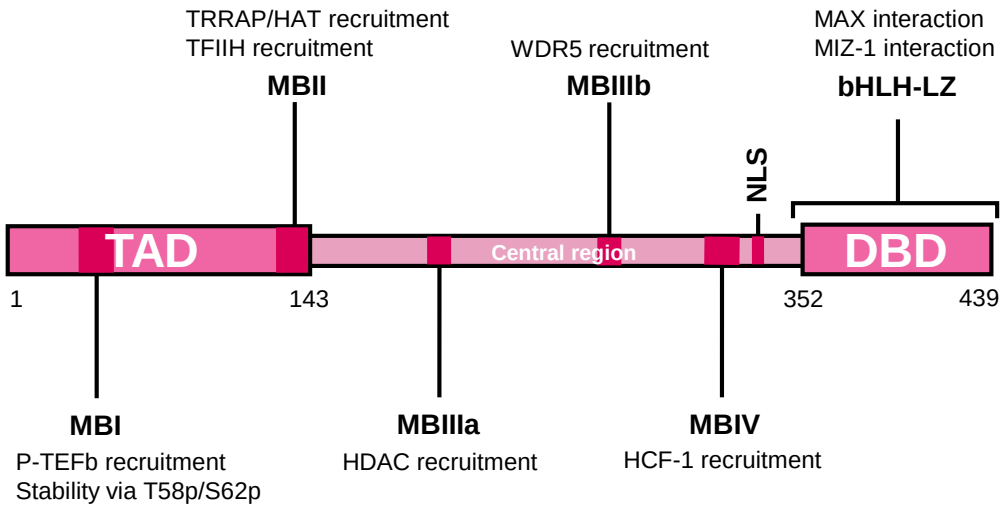

Figure 1.10: C-MYC protein architecture. Depicted is a schematic of the C-MYC protein encompassing its transactivation domain (TAD) and DNA-binding domain (DBD) relative to the MYC box (MB) subdomains. The main known functions of MB domains and the bHLH-LZ domain are also shown. T58p and S62p represents phosphorylation of corresponding residues. Numbers correspond to amino acid position. N-MYC has an analogous arrangement, whereas L-MYC lacks MBIIIa. NLS, nuclear localisation signal.

C-MYC knockout mouse embryos die by day 10.5 with stunted growth and hypoplastic/aberrantly developed organs (Davis et al., 1993). Deregulation of *C-MYC* – caused by increased *C-MYC* gene expression, protein stability and activity – can contribute to the initiation and progression of many solid and blood-borne tumours including those of the breast, prostate, gastrointestinal tract and many haematopoietic cancers. C-MYC regulates RNA pol I, II and III transcriptional activity. The genes regulated by C-MYC vary between cell types with the exception of a core signature which essentially supports cell growth and proliferation (Brown et al., 2008; Kress et al., 2015; McMahon, 2010) (see sections 1.4.4 and 1.4.5). Ultimately, C-MYC deregulation directly up- and down-regulates a subset of genes and causes amplification of general transcription and translation, increasing the propensity of cells to undergo oncogenic transformation.

Of note, most functional studies have focussed on *C-MYC* rather than the other *MYC* genes due to the overwhelming prevalence of *C-MYC* deregulation in cancers, although it is likely that at least some findings can be applied to all *MYC* members. *N-MYC* and *L-MYC* expression is subject to more spatial and temporal regulation than *C-MYC*, both during development and in adults. *N-MYC* is mostly expressed in neural tissue, kidney, heart and testes during development, where expression persists at low levels in adults (Jakobovits et al., 1985; Zimmerman et al., 1986). Amplification or overexpression of *N-MYC* is associated with the development of neuroendocrine cancers such as neuroblastomas, retinoblastomas, small cell lung cancers (SCLC) and prostate cancers (Lee et al., 2016b; Lee et al., 1984; Seeger et al., 1985; Wong et al., 1986). *N-MYC* knockout mice are embryonic

lethal, at a slightly later stage than *C-MYC* knockouts (~day 11.5) and similar to *C-MYC* knockouts are small in size while exhibiting developmental defects in several organs (Charron et al., 1992; Sawai et al., 1991; Stanton et al., 1992). It is thought that *C-MYC* and *N-MYC* knockout embryos survive to the stage they do because they can functionally compensate for each other (perhaps in concert with *L-MYC*) until the point when their expression patterns start to diverge. Indeed, *N-MYC* can functionally compensate for *C-MYC* knockout when the *N-MYC* coding sequence is placed in the endogenous *C-MYC* locus (Malynn et al., 2000). Taken together, *C-MYC* and *N-MYC* have similar molecular functions, but their distinct regulation of expression is important. In contrast to the other *MYC* family members, *L-MYC* knockout mice are viable and develop healthily as in WT mice (Hatton et al., 1996). *L-MYC* is generally co-expressed with *C-MYC* or *N-MYC* during development (Zimmerman et al., 1986), therefore it is thought that the other two *MYC* genes can functionally compensate for *L-MYC* loss. Additionally, L-MYC has a reduced capability to transactivate and induce cell transformation compared to C-MYC and N-MYC (Barrett et al., 1992; Birrer et al., 1988; Lee et al., 1985; Oster et al., 2003; Yancopoulos et al., 1985). During development, *L-MYC* expression is limited mainly to the central nervous system, kidneys and lungs with low level expression in other tissues (Hatton et al., 1996). *L-MYC* overexpression or amplification can also manifest in tumourigenesis, most commonly SCLC (Gu et al., 1988; Nau et al., 1985).

There are other, less characterised C-MYC isoforms arising from alternative start codons (p64 and p67), internal translation start sites (S-MYC), or proteolytic cleavage (MYC-nick) (Conacci-Sorrell et al., 2010; Hann and

Eisenman, 1984; Spotts et al., 1997; Stewart et al., 1984). For the most part, their functions are not clear, although more recent studies have shown that MYC-nick promotes cancer cell survival and invasion. MYC-nick lacks the DBD and nuclear localisation signal (NLS) and is thus located in the cytoplasm where it mediates cytoskeleton remodelling and promotes autophagy rather than apoptosis, lending to the above effects (Anderson et al., 2016; Conacci-Sorrell et al., 2010).

1.4.3 C-MYC-MAX dynamics

MAX is the primary partner of C-MYC in transcriptional regulation, and is required for both gene activation and repression by C-MYC (Amati et al., 1992; Cowling and Cole, 2007b; Mao et al., 2003). Moreover, the interaction is essential for many aspects of C-MYC biology and for its full transformative potential (Amati et al., 1993a; Amati et al., 1993b). C-MYC-MAX dimers can form tetramers which co-operatively bind chromatin and facilitate promoter DNA bending (Lebel et al., 2007; Nair and Burley, 2003), although its significance in vivo is yet to be explored. *MAX* knockout is lethal in mouse embryos at a significantly earlier stage (day 5.5-6.5) compared to *C-MYC* and *N-MYC* knockout mice (Shen-Li et al., 2000), which likely owes to lack of a compensatory factor unlike the three *MYC* genes. Endogenous *MAX* may be essential at even earlier stages of development (pre-implantation), since embryonic lethality coincides with loss of stable maternal MAX protein in the embryo (Shen-Li et al., 2000). *C-MYC* expression is highly dynamic (discussed in section 1.4.9), whereas *MAX* is constitutively expressed in stoichiometric excess to *C-MYC* with its mRNA and protein products being more stable and subject to less regulation (Berberich et al., 1992; Blackwood et al., 1992;

Wagner et al., 1992). However, a number of MAX binding partners exist other than C-MYC to antagonise C-MYC-MAX. MAX can form weak homodimers (when expressed highly enough relative to C-MYC), in addition to heterodimers with other bHLH-LZ transcription factors: MAX dimerization proteins (MXD) 1-4, MAX network transcriptional repressor (MNT) and MAX gene-associated (MGA) (Kretzner et al., 1992). All complexes have the same DNA binding specificity as C-MYC-MAX dimers, causing competition for promoter region binding. Alternative MAX complexes not only block C-MYC recruitment and C-MYC's ability to activate transcription, but they can also recruit histone deacetylases/methyltransferases; passively or actively repressing genes, respectively (Hurlin et al., 1997; Laherty et al., 1997; Ogawa et al., 2002; Yin et al., 1998). Indeed, although moderate expression of MAX enhances C-MYC-induced cell transformation, MAX overexpression actually antagonises C-MYC-induced cell transformation in a dose-dependent manner (Amati et al., 1993a; Makela et al., 1992). In proliferating cells, MAX exhibits preference towards C-MYC for dimerisation, whereas upon differentiation other interactions are favoured (Ayer and Eisenman, 1993; Blackwell et al., 1990; Prendergast and Ziff, 1991). MAX-interacting partners have opposite biological roles to that of C-MYC with regard to target gene activation and cell transformation (Hurlin et al., 1999; Kato et al., 1992; Nilsson et al., 2004; Schreiber-Agus et al., 1998). Taken together, when MAX is in a heterodimer with C-MYC it acts as a transcription co-factor of C-MYC, but when engaged in alternative complexes it antagonises C-MYC function.

1.4.4 C-MYC-dependent gene regulation: global or specific?

C-MYC regulates thousands of genes (10-30% of the genome) which mostly vary depending on the cell type and conditions except a 'core signature' of genes involved in RNA processing, ribosome biogenesis and biomass accumulation (Fernandez et al., 2003; Ji et al., 2011; Li et al., 2003b; Zeller et al., 2006). Although C-MYC preferentially binds E-boxes in vitro and exhibits some preference of E-boxes in vivo, C-MYC genome binding in cells correlates more with RNA pol II location than E-boxes (Guccione et al., 2006; Guo et al., 2014). It was proposed that C-MYC binds and regulates all transcriptionally active genes and enhancers, serving as an amplifier of transcriptional output and therefore upregulating the existing gene expression programme, rather than inducing a new set of genes (Lin et al., 2012b; Nie et al., 2012). This is consistent with the 'core signature' of C-MYC target genes being expressed in all cell types (Ji et al., 2011). More recently, it was shown that C-MYC transcriptional regulation and gene expression amplification can be separable events, indicating that C-MYC binds and regulates a distinct set of genes while indirectly amplifying gene expression through its target genes involved in global regulation of gene expression (Sabo et al., 2014; Walz et al., 2014). Mathematical modelling has postulated that genes with high affinity C-MYC binding sites (E-boxes) are upregulated even in the presence of low C-MYC levels, whereas those with low-affinity C-MYC binding sites (non-canonical E-boxes or non-specific DNA sequences, i.e. global transcription amplification) are only upregulated upon C-MYC overexpression (Figure 1.9) (Benary et al., 2016; Lorenzin et al., 2016). Although a unified theory is yet to be solidified, it is evident that C-MYC target genes can vary between systems depending on C-MYC levels, RNA pol II status, chromatin context and the stoichiometry of C-

MYC interacting partners. A few genes which are ascribed as C-MYC targets in many systems will be described in the following section, and their expression will be analysed in particular parts of this thesis. These C-MYC target genes are involved in ribosome biogenesis, metabolism, chromatin modification, translation and the cell cycle.

1.4.5 C-MYC target genes

Nucleolin (*NCL*), nucleophosmin (*NPM1*) and fibrillarin (*FBL)* encode abundant nucleolar proteins which are transcriptionally regulated by C-MYC (Greasley et al., 2000; Ji et al., 2011; Zeller et al., 2001; Zeller et al., 2003). They have roles in promoting ribosome biogenesis at various stages, including RNA pol I transcription initiation and elongation (at ribosomal DNA, rDNA), processing of rRNA and assembly of rRNA with ribosomal proteins (Cong et al., 2012; Ginisty et al., 1998; Murano et al., 2008; Rickards et al., 2007; Savkur and Olson, 1998; Tollervey et al., 1993). NCL, NPM1 and FBL are often upregulated in cancers, (Berger et al., 2015; Holmberg Olausson et al., 2015; Koh et al., 2011; Lim and Wang, 2006; Marcel et al., 2013; Su et al., 2014), likely supporting aberrant growth and proliferation of tumour cells. Intriguingly, when NCL is overexpressed it can also be found on the cell surface where it acts to transduce oncogenic and suppress anti-oncogenic signals (Reyes-Reyes and Akiyama, 2008; Wise et al., 2013).

Ornithine decarboxylase (*ODC*) is a C-MYC target gene encoding an enzyme rate-limiting in polyamine synthesis (Bello-Fernandez et al., 1993; Zeller et al., 2003). Polyamines can bind DNA, RNA and proteins, thereby influencing DNA/chromatin structure and stability, RNA processing, translation

and protein activity (Childs et al., 2003; Jänne et al., 2004). ODC and polyamine levels are important in the regulation of processes such as cell proliferation, cellular stress responses and apoptosis (Jänne et al., 2004; Miller-Fleming et al., 2015). ODC overexpression in itself is sufficient for cell transformation and occurs in various cancers (Auvinen et al., 1992; Deng et al., 2008; Hu et al., 2005; Tamori et al., 1995). Another metabolism-associated C-MYC-target gene is nucleoside diphosphate kinase A/non-metastatic cells 1 (*NME1*), encoding an enzyme which utilises ATP to generate other nucleoside triphosphates. Additionally, NME1 phosphorylates and activates kinase suppressor of RAS (KSR), thereby down-modulating oncogenic RAS signalling (Hartsough et al., 2002). NME1 also possesses exonuclease activity which is important for DNA replication and repair (Jarrett et al., 2012; Ma et al., 2004). Not surprisingly, NME1 is a suppressor of metastasis (Kaetzel et al., 2006; McCorkle et al., 2014; Steeg et al., 1988).

TIP49 (TATA box-binding protein-interacting protein 49kDa) is a DNA helicase and an essential subunit of the C-MYC co-activator tat-interactive protein 60kDa (TIP60) histone acetyltransferase (HAT) complex (discussed in section 1.4.6) (Jha et al., 2008). It is also transcriptionally induced by C-MYC (Cowling and Cole, 2007b; Westermann et al., 2008; Wood et al., 2000). The TIP60 complex is essential for C-MYC target gene expression and C-MYC-mediated cell transformation (Wood et al., 2000).

Components of the eIF4F complex (which mediates cap-dependent translation) are transcriptionally upregulated by C-MYC and contribute to C-MYC-dependent translation and protein synthesis (Jones et al., 1996; Lin et al., 2008; Rosenwald et al., 1993b). This exists as part of a positive feedback loop

whereby C-MYC increases eIF4F expression and in turn eIF4F upregulates C-MYC expression and activity (Lin et al., 2008). The eIF4E cap-binding component in itself, or in cooperation with C-MYC, promotes cell transformation and oncogenesis (Avdulov et al., 2004; Li et al., 2003a; Polunovsky et al., 1996; Ruggero et al., 2004).

The *CCND1* gene, encoding cyclin D1, can be positively or negatively regulated by C-MYC depending on the system (Daksis et al., 1994; Huerta et al., 2007; Philipp et al., 1994; Rosenwald et al., 1993a), possibly through context-dependent positive or negative transactivators. For example, *CCND1* is repressed by C-MYC and histone deacetylases (HDACs) in a complex involving zona occludens 2 (ZO-2) – a tight-junction protein with roles in intercellular communication, found in the cell membrane but also in the nucleus (Huerta et al., 2007). Cyclin D1 is the regulatory subunit of CDK4 and CDK6, and promotes the G1-S transition in the cell cycle (Resnitzky et al., 1994). Overexpression of cyclin D1 causes aberrant cell proliferation and often occurs in breast cancers amongst others (Musgrove et al., 2011; Wang et al., 1994).

1.4.6 Transcription activation by C-MYC

C-MYC activates transcription by recruiting HAT complexes and RNA pol II kinases, causing chromatin relaxation and influencing RNA pol II pausing, respectively. C-MYC directly associates with particular subunits of several HAT complexes through its TAD, which increases C-MYC target gene histone acetylation at histones 3 and 4 (H3/4) (Frank et al., 2001; Martinato et al., 2008). Specifically, C-MYC interacts with transformation-TAD-associated protein (TRRAP) and other HAT complex proteins to recruit different histone

acetyltransferases: general control of amino-acid synthesis 5 (GCN5) or TIP60 (Frank et al., 2003; Liu et al., 2003; McMahon et al., 1998; McMahon et al., 2000). TRRAP, GCN5 and TIP60 are required for C-MYC-dependent transcriptional activation and transformation (Flinn et al., 2002; Liu et al., 2003; McMahon et al., 2000; Wood et al., 2000). C-MYC also recruits the p300 and CBP HATs in a distinct complex which facilitates C-MYC-mediated transcription, with p300 binding the C-MYC TAD and CBP binding the DBD (Faiola et al., 2005; Vervoorts et al., 2003). More recently, C-MYC was shown to bind to WD40-repeat protein 5 (WDR5) which is important for C-MYC DNA binding (Thomas et al., 2015b), and host cell factor 1 (HCF-1) scaffold protein which is known to recruit a range of different chromatin modifiers (Thomas et al., 2016). C-MYC recruits different chromatin modifier complexes in different studies, and to date there is no unifying model of recruitment specificity. It is thought that particular complexes might regulate specific genes depending on the circumstances.

Additionally, C-MYC mediates transcription activation by increasing the expression and recruitment of TFIIH and P-TEFb complexes (which contain CDK7 and CDK9, respectively) to promoters, which is dependent on the C-MYC TAD domain but not the DBD (Bouchard et al., 2004; Cowling and Cole, 2007b; Kanazawa et al., 2003; Mateyak et al., 1999). As previously mentioned, CDK7 and CDK9 phosphorylate S5 and S2 of the RNA pol II CTD, respectively, which in concert co-ordinate the recruitment of factors involved in RNA pol II pausing, pause release, elongation, mRNA processing and termination (Hsin and Manley, 2012). C-MYC (and N-MYC) modulation correlates with global cellular levels of S5- and S2- phosphorylated RNA pol II (Cowling and Cole, 2007b; Lin

et al., 2012b). In most cases, C-MYC induces RNA pol II pause release without affecting RNA pol II initiation, in a P-TEFb-dependent manner (Eberhardy and Farnham, 2002; Lin et al., 2012b; Nie et al., 2012; Rahl et al., 2010). However, in a particular study C-MYC was shown to regulate transcription initiation as well as elongation (Walz et al., 2014), although the mechanisms governing this are not yet clear. It may be that C-MYC fulfils different roles in transcription depending on its abundance, the presence of different initiation/elongation factors, or on the chromatin environment. Moreover, as previously discussed, C-MYC upregulates mRNA capping in concurrence with increased RNA pol II phosphorylation (Cowling and Cole, 2007b; Posternak et al., 2017). Intriguingly, although the C-MYC DBD and C-MYC's interaction with MAX were required for C-MYC target gene activation, they were not required for C-MYC-induced RNA pol II phosphorylation and mRNA capping, and C-MYC mutants lacking these regions were able to confer a partial C-MYC phenotype (Cowling and Cole, 2007b). Perhaps when C-MYC is above a certain concentration in the nucleus it can reach RNA pol II complexes without direct DNA binding, although the DBD likely provides specificity in some contexts. It is not yet clear if C-MYC recruits HATs and CDKs to genes in a synergistic manner to drive transcriptional activation, whether CDK recruitment occurs as a consequence of C-MYC-mediated histone acetylation, or if HAT and CDK recruitment are each preferentially recruited to specific genes. Further studies are required to dissect the relative importance and specificity of these C-MYC transactivation modules.

In addition to regulating RNA pol II transcription, C-MYC also transactivates RNA pol I- and III- transcribed genes (Arabi et al., 2005; Gomez-Roman et al., 2003; Grandori et al., 2005; Poortinga et al., 2004b). As

previously mentioned, RNA pol I synthesises 45S rRNA whereas RNA pol III synthesises 5S rRNA and transfer RNA (tRNA). These components are essential for ribosome biogenesis and mRNA translation. RNA pol I and III are required for cell growth, especially in cancer cells which exhibit aberrant proliferation and growth control mechanisms. C-MYC influences RNA pol I and III transcription in many ways. Firstly, C-MYC directly transactivates RNA pol II-transcribed genes encoding RNA pol I and III subunits, transactivators and RNA processing factors (Gomez-Roman et al., 2003; Ji et al., 2011; Poortinga et al., 2011; Schlosser et al., 2003). C-MYC also directly binds to RNA pol I- and III-transcribed genes and influences their transcription. In the case of RNA pol I transcription, C-MYC increases the recruitment of selective factor 1 complex (SL1, important for pre-initiation complex recruitment) and increases RNA pol I loading (Arabi et al., 2005; Grandori et al., 2005). On RNA pol III-transcribed genes, C-MYC recruits TFIIB (transcription initiation factor) and increases RNA pol III loading (Koch et al., 2007; Steiger et al., 2008). Moreover, C-MYC recruits HATs via TRRAP to create a permissive chromatin environment for RNA pol III transcription as described for RNA pol II (Arabi et al., 2005; Grandori et al., 2005; Kenneth et al., 2007). On the *RNA45S5* gene (encoding 45S precursor rRNA), C-MYC also influences rDNA looping to promote efficient RNA pol I transcription (Shiue et al., 2009). It is not known whether C-MYC also promotes higher order chromatin structures on RNA pol I-transcribed genes. Of note, RNA pol I and III do not possess a CTD ‘tail’ structure like that of RNA pol II, therefore it is thought that they are not amenable to C-MYC-dependent CDK7/9 activity. Ribosome biogenesis is critical to C-MYC function, since mouse models of C-MYC-driven Burkitt’s lymphoma require both copies of the

gene encoding L24 ribosomal protein for C-MYC's full oncogenic potential (Barna et al., 2008).

1.4.7 Transcription repression by C-MYC

C-MYC is also able to directly repress the expression of particular genes. Although this is less well understood than C-MYC-induced transcription, it is thought to occur through displacement of transactivators and recruitment of transcriptional repressors. Notably, the first identified C-MYC-repressed gene was *C-MYC* itself, creating a negative feedback loop and tumour-suppressor mechanism when C-MYC expression reaches a critical threshold (Adams et al., 1985; Murphy et al., 2008; Penn et al., 1990). One mechanism of C-MYC-mediated gene repression is via MYC-interacting zinc finger protein (MIZ-1). MIZ-1 is a soluble protein able to activate the transcription of certain genes (including those which regulate the cell cycle, cell growth and apoptosis) in a C-MYC-independent manner by recruiting HATs (Staller et al., 2001a; Varlakhanova et al., 2011; Ziegelbauer et al., 2004). However, when C-MYC interacts with MIZ-1, MIZ-1 becomes insoluble and instead recruits DNA methyltransferase 3 alpha (DNMT3A) to promoter regions, creating a restrictive chromatin environment, shutting down the expression of C-MYC-MIZ-1 target genes and thus alleviating cell cycle and growth restraints (Brenner et al., 2005; Peukert et al., 1997; Staller et al., 2001a; Varlakhanova et al., 2011). Of note, a single point mutation in C-MYC which disrupts its interaction with MIZ-1 diminishes its oncogenic potential (Van Riggelen et al., 2010a), demonstrating the importance of this mechanism. C-MYC and N-MYC can also interact with specificity protein 1 (SP1) – a zinc finger transcription factor – thereby suppressing SP1 target genes including the gene encoding CDK inhibitor p21.

To achieve this, C-MYC sequesters SP1 from promoters or recruits HDACs (Gartel et al., 2001b; Liu et al., 2007). Interestingly, C-MYC can also negatively regulate genes in an E-box-dependent manner by recruiting HDACs, which for example occurs on the genes encoding CDK inhibitor p27 and cyclin D1 (Amente et al., 2011; Chandramohan et al., 2008; Huerta et al., 2007; Kurland and Tansey, 2008). The mechanisms governing whether C-MYC activates or represses a given E-box-associated gene are not clear, although it may involve differential expression or availability of transcriptional co-factors.

In addition to direct transcriptional repression, C-MYC can also achieve this via indirect mechanisms. C-MYC induces the expression of some miRNAs, thereby inhibiting translation of particular mRNAs or inducing their degradation. One such example is the C-MYC-induced miRNA cluster *miR-17-92* (encoding six miRNAs) which cooperates with C-MYC in B cell lymphomagenesis (He et al., 2005; O'Donnell et al., 2005). In a further layer of complexity, C-MYC can directly repress other miRNA-encoding genes, which contributes to the oncogenic potential of lymphoma cells (Chang et al., 2008). Moreover, C-MYC indirectly causes activation and stabilisation of the methyltransferase enhancer of zeste 2 (EZH2) which trimethylates lysine 27 on H3 (H3K27me3) causing epigenetic silencing (Kaur and Cole, 2013). In at least certain systems this mediates widespread gene repression.

1.4.8 Tumour-relevant functions of C-MYC

C-MYC has been implicated in the control of essentially all cellular processes relevant to cancer initiation, development and maintenance in one setting or another. Several of these have been mentioned already, but for

clarification will be highlighted here (Figure 1.11). C-MYC drives DNA replication and the cell cycle, enforcing rapid cell division in the absence of appropriate mitogens (Bretones et al., 2015). Consequently, C-MYC causes replicative stress and DNA damage (Dominguez-Sola and Gautier, 2014). Cell growth and metabolism are regulated by C-MYC to provide sufficient nutrients, energy and proteins for the maintenance of tumour cell proliferation (Campbell and White, 2014; Schmidt et al., 2009; Stine et al., 2015). C-MYC can promote apoptosis as an intrinsic tumour suppressor mechanism (McMahon, 2014). Finally, C-MYC decreases cell adhesion, promotes cell motility and encourages tumour angiogenesis, thus supporting tumour maintenance and metastasis (Wolfer and Ramaswamy, 2011). It is not clear if all of these mechanisms in cohort are important for C-MYC oncogenic function, or whether dysregulation of particular processes are more important in different cancer types.

1.4.9 Homeostatic regulation and tumourigenic deregulation of C-MYC

In non-oncogenic contexts, C-MYC is regulated at essentially every possible opportunity to maintain appropriate cell proliferation and prevent neoplasia (Figure 1.12). *C-MYC* is expressed at low levels in quiescent cells, but is rapidly induced in response to growth factor signalling (Dean et al., 1986). The *C-MYC* gene is influenced by regulation of both transcription initiation and elongation by a range of transcription factors (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Krystal et al., 1988). *C-MYC* mRNA nuclear export and translation initiation are positively regulated by eIF4E (Culjkovic et al., 2006; Lin et al., 2008), and its translation can be positive or negatively regulated by

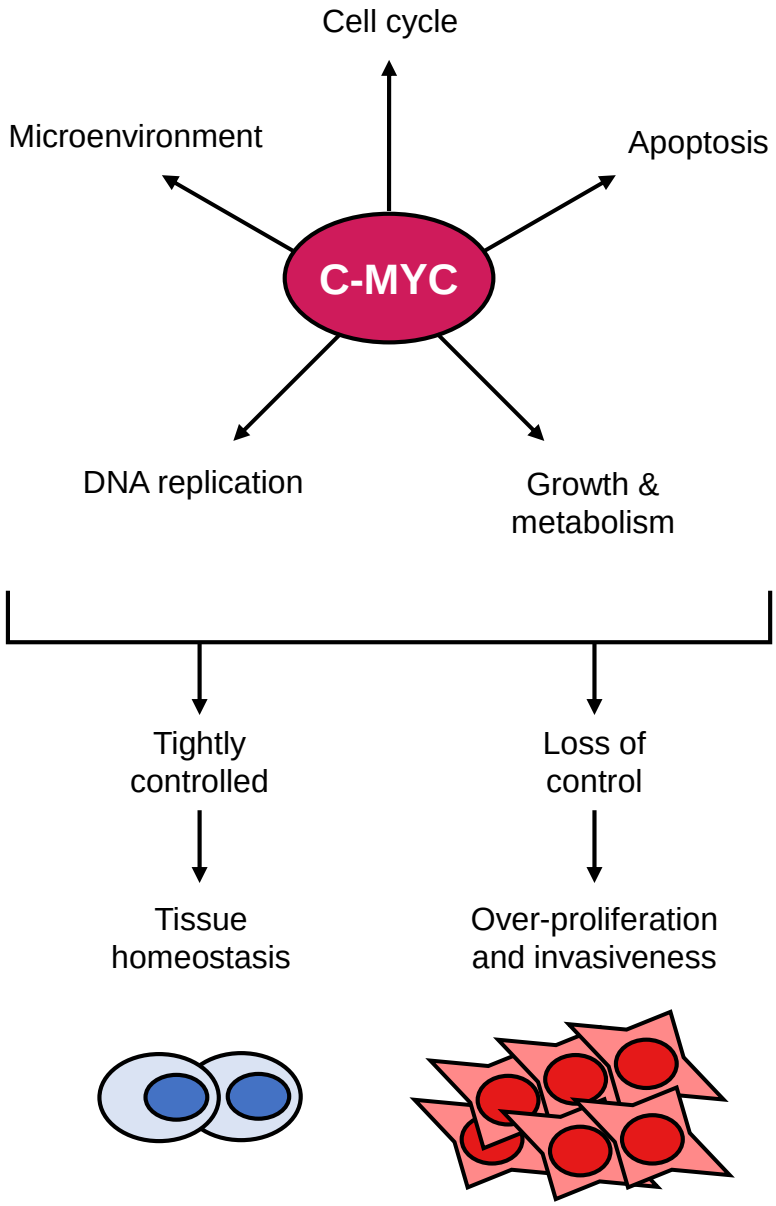

Figure 1.11: Cellular functions of C-MYC. Some main cellular functions controlled by C-MYC are shown here, and described in more detail within the text. C-MYC functions in normal conditions to control appropriate cell behaviour, but when deregulated has roles in tumour initiation, maintenance and development.

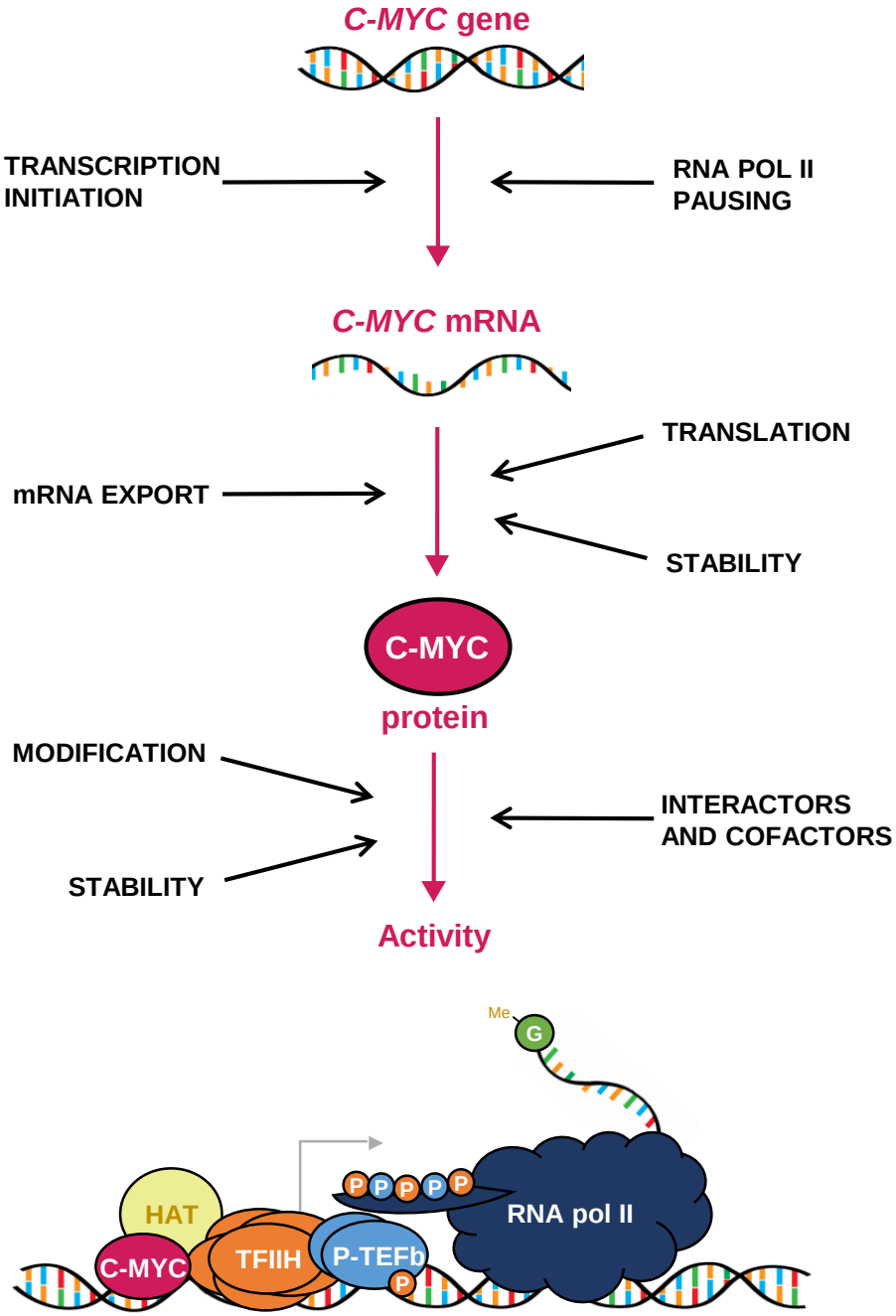

Figure 1.12: Extensive C-MYC regulation. Mechanisms of C-MYC regulation are shown. At essentially every possible stage of C-MYC expression, it is subject to positive or negative regulation depending on cellular stimuli. These processes are utilised in normal cells to retain proper control of C-MYC, whereas in cancer cells they are often perturbed, resulting in increased C-MYC expression or activity. Specific examples are discussed in the text.

specific RNA-binding proteins (Filipenko et al., 2004; Lemm and Ross, 2002; Mazan-Mamczarz et al., 2006). Furthermore, *C-MYC* mRNA is intrinsically unstable with a typical half-life of 10-30 minutes (Dani et al., 1984; Laird-Offringa et al., 1991; Welcker et al., 2004), but it can be stabilised by RNA-binding proteins (Lemm and Ross, 2002; Prokipcak et al., 1994). The C-MYC protein is also unstable (with a typical half-life of 20-30 minutes) (Hann and Eisenman, 1984; Ramsay et al., 1984), and is influenced by dynamic patterns of post-translational modifications including phosphorylation, ubiquitination, acetylation and glycosylation which control its stability and activity (Chou et al., 1995; Liu et al., 2013; Noguchi et al., 1999; Patel et al., 2004; Popov et al., 2007; Sears et al., 2000; Vervoorts et al., 2003; Welcker et al., 2004). Moreover, as previously discussed, C-MYC transcriptional activity is subject to regulation via its interacting partners and co-factors.

C-MYC is overexpressed in approximately 50% of all cancers (Vita and Henriksson, 2006). C-MYC is most commonly upregulated or hyperactivated via altered upstream signal transduction. Many signalling pathways known to be deregulated in cancer culminate in C-MYC activation via upregulation of its expression or activity as described above. Oncogenic/tumour suppressive pathways that regulate C-MYC include adenomatous polyposis coli (APC) signalling, which suppresses C-MYC and is defective in colon cancers (He et al., 1998); NOTCH signalling which is upregulated in haematopoietic malignancies and activates *C-MYC* expression (Palomero et al., 2006; Weng et al., 2006); and the RAS- phosphoinositide 3-kinase (PI3K)/ERK pathways which promote phosphorylation of C-MYC at two sites; governing concomitant C-MYC activation and destabilisation (Sears et al., 2000). C-MYC Threonine 58 (T58)

phosphorylation by glycogen synthase kinase 3 (GSK-3) mediates its ubiquitination and proteasomal degradation, whereas C-MYC Serine 62 (S62) phosphorylation by ERK paradoxically stabilises the protein while priming it for phosphorylation at T58 (Sears et al., 2000).

The *C-MYC* gene is amplified in a range of tumour types (Vita and Henriksson, 2006). Although less common, the *C-MYC* gene can also be upregulated by changes in its regulatory regions or coding sequence, which are more specifically associated with Burkitt's lymphoma and AIDS-associated haematopoietic cancers (Bhatia et al., 1993; Bhatia et al., 1994; Hemann et al., 2005). In B cells, *C-MYC* can be translocated to the immunoglobulin heavy chain locus, causing it to be constitutively active under control of an associated enhancer region (Adams et al., 1985). As previously mentioned, *C-MYC* can also be activated by retroviral insertion (Adey et al., 2013; Hayward et al., 1981), leading to elevated C-MYC expression. An oncogenic mutation encoding a Threonine-Alanine alteration at position 58 (T58A) prevents phosphorylation of C-MYC at this residue, which as previously mentioned promotes wild-type C-MYC degradation (Hemann et al., 2005). Consequently, the C-MYC T58A variant is substantially stabilised, leading to greater activity and oncogenic potential (Bahram et al., 2000; Wang et al., 2011).

1.4.10 C-MYC response thresholds

The phenomenon of distinct C-MYC expression thresholds governing different cellular responses has been documented in several studies, predominantly in cancer systems but also in development. Cells can be very sensitive to even small alterations in C-MYC levels. For example, disruption of a

C-MYC regulatory region (containing a common cancer-associated single nucleotide polymorphism) in mouse models only marginally lowered *C-MYC* mRNA levels and caused no obvious adverse effects on the animals, whilst conferring resistance to APC-induced intestinal polyps (Sur et al., 2012). In another study, mouse models overexpressing C-MYC in haematopoietic cells to different extents developed different tumour types depending on C-MYC levels: those expressing the highest levels of C-MYC developed aggressive T-cell lymphomas and those with lower levels developed late-onset monocytic tumours (Smith et al., 2006). Mice with intermediate C-MYC expression levels developed different proportions of these tumour types, corresponding to C-MYC levels. It is thought that increased C-MYC expression increases the propensity of cells to accrue gain-of-function mutations in anti-apoptotic genes which synergise with C-MYC in T-cell lymphomagenesis, which could account for the development of different tumour types.

As previously discussed, C-MYC paradoxically promotes cell growth and proliferation, while also priming cells for apoptosis as an intrinsic tumour suppressor mechanism. The balance of these conflicting cellular processes is governed by distinct levels of C-MYC expression or activity. For example, a version of C-MYC which is unresponsive to growth signals (but expressed at a similar level to mitogen-stimulated endogenous C-MYC) was sufficient to confer an increase in proliferation of regularly cycling somatic cells. However, in the same system, gross C-MYC overexpression induced concomitant cell proliferation and apoptosis (Murphy et al., 2008). Conversely, in Drosophila wing development, cells engineered to express *dMyc* (Drosophila *C-MYC* homologue) just two-fold higher than WT levels over-proliferated, whilst those

expressing two-fold less than WT underwent apoptosis (Moreno and Basler, 2004). The apoptotic response to C-MYC in some cases is likely influenced by the balance of pro-/anti-apoptotic proteins and survival factors (Askew et al., 1991; Evan et al., 1992; Hoffman and Liebermann, 2008). Indeed, co-deregulation of *C-MYC* with apoptosis-associated genes such as B-cell lymphoma 2 (*BCL-2*), BCL-2-like protein 11 (*BIM*), and *p53* overrides C-MYC-induced apoptosis and augments tumourigenesis (Hemann et al., 2005; Nesbit et al., 1998).

Moreover, there are many genes and pathways which are not required for cell viability under normal conditions that become essential upon deregulation of C-MYC (examples discussed in section 1.5.6). The cause for particular thresholds of C-MYC expression/activity governing distinct cellular outcomes (rather than simply acting on an axis; enhancing or diminishing one outcome) could be due to C-MYC binding and regulating additional sets of genes when the concentration of C-MYC in the nucleus is above a certain level. The availability of specific co-factors involved in C-MYC-mediated transcription may also play a role.

1.5 Therapeutic intervention of C-MYC in cancer

1.5.1 Targeting C-MYC protein function

With the overwhelming evidence of C-MYC's involvement in cancer, much effort has been invested in trying to find and develop C-MYC inhibitors to use as chemotherapeutic agents. One route that has been explored is directly inhibiting the function of the C-MYC protein. To determine if this was a viable option, a peptide called Omomyc was developed, which forms homodimers and binds particular E-boxes with a greater affinity than C-MYC-MAX, thus blocking C-MYC-induced transactivation on those genes (Jung et al., 2016; Soucek et al., 2002) . Conditionally expressing Omomyc in mouse models of *KRAS*-induced lung cancer caused tumour regression, while causing some adverse yet reversible side-affects in proliferating somatic tissues (Soucek et al., 2008). This is proof of concept that directly inhibiting C-MYC function could be an effective mode of action for anti-cancer therapies. However, the C-MYC protein lacks a defined active site, making it problematic to target using small molecule inhibitors. In addition, the structure of monomeric MYC is intrinsically disordered relative to when it is in a heterodimer (Fieber et al., 2001). As an alternative, inhibitors which disrupt the interaction of C-MYC with its binding partner MAX, and those which disrupt C-MYC-MAX DNA binding, have been discovered from chemical library screens with the intention of depleting C-MYC transcriptional activity (Berg et al., 2002; Kiessling et al., 2006; Wang et al., 2007; Yin et al., 2003). However, relatively high concentrations were required to perturb the C-MYC-MAX interaction and to inhibit cell growth. Inhibitors of C-MYC-MAX chromatin recruitment were tested in human xenograft mouse models, but were unstable in vivo, were not effectively localised to tumours, and displayed no

anti-tumour activity (Clausen et al., 2010; Guo et al., 2009). The development of more efficacious C-MYC-MAX dimerisation and DNA binding inhibitors is in progress (Jung et al., 2015; Wang et al., 2013a; Wanner et al., 2015). Taken together, inhibiting C-MYC protein function could be an effective mode of action for a chemotherapy, however more work is required to optimise drugs which can do so in vivo.

1.5.2 Targeting *C-MYC* expression

Since directly perturbing C-MYC function is problematic, alternative methods are being explored, including targeting the expression of *C-MYC*. Studies using mouse models of cancer conditionally expressing *C-MYC* provide rationale that inactivating *C-MYC* expression is an effective way to achieve sustained tumour regression (Jain et al., 2002; Shachaf et al., 2004). Inhibition of the BET family of proteins has been found to inhibit C-MYC expression (Dawson et al., 2011; Delmore et al., 2011; Zuber et al., 2011) and small molecule BET inhibitors are showing some positive therapeutic effects in clinical trials (Berthon et al., 2016; Stathis et al., 2016). However, BET inhibition interferes with the expression of many genes and does not always perturb C-MYC expression (Baker et al., 2015; Fong et al., 2015), suggesting there could be tissue-specific regulation of *C-MYC* by BET proteins or compensatory mechanisms may occur to maintain C-MYC expression. In favour of the latter, studies in models of acute myeloid leukaemia demonstrated that *C-MYC* expression was either maintained or recovered after BET inhibition (Fong et al., 2015; Rathert et al., 2015). Moreover, it was shown that interfering with *C-MYC* expression did not recapitulate the effect of BET inhibition on cell viability (Ambrosini et al., 2015).

Another potential therapeutic strategy for targeting *C-MYC* expression is stabilising G-quadruplexes in the *C-MYC* promoter, which negatively regulate transcription of the *C-MYC* gene. One small molecule compound which binds to the *C-MYC* G-quadruplex (Quarfloxin/CX-3543) was taken forward to phase II clinical trials, but its mechanism of action was actually shown to be through stabilising these structures on rRNA genes and had no effect on *C-MYC* expression (Drygin et al., 2009).

Inhibition of CDK7 – which controls C-MYC expression and function – using an inhibitor called THZ1 specifically targeted small cell lung cancer, neuroblastoma and triple-negative breast cancer cells which harbour amplified or deregulated MYC family members (Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Wang et al., 2015). Efficacy was likely due to THZ1 reducing super-enhancer-driven expression of *MYC* genes and other oncogenes exclusively in cancer cells but not normal cells (Figure 1.9). Super-enhancers are clusters of enhancers bound by hubs of transcription apparatus and promote strong activation of effector genes, which was thought to render *C-MYC* and other genes particularly sensitive to CDK7 inhibition.

Similar to CDK7, CDK9 promotes C-MYC expression and activity. CDK9 inhibition was shown to be effective in hepatocellular carcinoma models selectively in the presence of C-MYC overexpression (Huang et al., 2014). However, despite inducing global RNA pol II pausing defects, CDK9 inhibition induced compensatory C-MYC expression through increased BET-bromodomain 4 (BRD4) -mediated CDK9 recruitment to the *C-MYC* gene (Lu et al., 2015). Furthermore, BET inhibition decreased CDK9-dependent elongation

of C-MYC target genes in B cell cancer cells, but resistance was conferred in some cases by compensatory RNA pol II loading (Donato et al., 2016).

A relatively novel strategy for oncogene inhibition is delivering specific siRNA molecules to tumours via nanoparticles, which is being tested in phase I clinical trials against various cancers (Young et al., 2016). Delivering *C-MYC* siRNA to tumours in via nanoparticles decreased, but did not halt, cancer progression in pre-clinical models (Chen et al., 2010a; Chen et al., 2010b) although co-delivery with the nucleoside analogue gemcitabine monophosphate (GMP, a currently available chemotherapy) improved the anti-tumour response (Zhang et al., 2013).

In summary, there is some evidence that interfering with *C-MYC* expression could be an effective anti-cancer strategy, and there are drugs currently available which attenuate *C-MYC* expression in at least some circumstances. However, resistance to chemotherapy could be a problem since compensatory or redundant mechanisms are in place to control *C-MYC* expression. Perhaps combination therapies are required in order for this strategy to be successful.

1.5.3 Targeting signalling upstream of C-MYC

As discussed in section 1.4.9, C-MYC is subject to extensive regulation by cellular signalling pathways on multiple levels. Targeting signalling upstream of C-MYC by has been explored as a potential route for tumour intervention. Interestingly, amplification or overexpression of C-MYC confers resistance to chemical inhibition of the PI3K pathway in breast and prostate cancer cells, (Carver et al., 2011; Ilic et al., 2011; Muellner et al., 2011). C-MYC confers

heightened cellular protein translation, which may override decreased translation upon PI3K inhibition. Indeed, combined inhibition of BET proteins in combination with PI3K depleted N-MYC expression and showed promising results in N-MYC-driven models of neuroblastoma (Andrews et al., 2017). Moreover, dual inhibition of mTOR complex 1 (mTORC1) within the PI3K pathway and RNA pol I exerts anti-tumour effects in C-MYC-driven B cell lymphoma models (Devlin et al., 2016). Resistance to PI3K pathway inhibition in this case might be subverted by diminishing the ability of N-MYC to regulate ribosome biogenesis and therefore translation. Alternatively, directly antagonising NOTCH-mediated transcriptional activation perturbs *C-MYC* expression, decreases the expression of C-MYC target genes and perturbs tumour progression in mouse models of T-cell acute lymphoblastic leukaemia (Moellering et al., 2009). Understanding the interplay of signalling pathways with C-MYC and with each other in different cancer types will help elucidate which pathways to best target for suppression of C-MYC-mediated oncogenesis.

1.5.4 Targeting C-MYC target genes

Since C-MYC functions through such diverse mechanisms, it is difficult to imagine how targeting individual or even combinations of C-MYC target genes could perturb C-MYC oncogenicity. There are however some C-MYC targets which have been considered as potential therapeutic targets; albeit not always in the context of C-MYC upregulation. One interesting example is targeting NCL on the surface cells via an immunoagent, which is co-internalised with NCL and prevents it from binding RNA substrates, thereby reducing the proliferation of some breast cancer cell lines (Palmieri et al., 2015). ODC inhibition has shown anti-tumour activity in C-MYC/N-MYC-driven myeloproliferative disorders/

neuroblastomas (Funakoshi-Tago et al., 2013; Hogarty et al., 2008). However, as one might expect, ODC is not required for all C-MYC functions including C-MYC-mediated differentiation blockade (Selvakumaran et al., 1996). In another study, knockdown of NPM1 in combination with RNA pol II inhibition (but not NPM1 knockdown alone) induced glioblastoma cell death (Holmberg Olausson et al., 2015). Components of the eIF4F complex, which are transcriptionally regulated by C-MYC, are also being considered as therapeutic targets in C-MYC-driven cancer cells (see section 1.5.6). In conclusion, inhibiting C-MYC target genes in cancer cells could have limited or context-dependent efficacy, and may require the use of combination therapies.

1.5.5 C-MYC co-factors and effectors as potential therapeutic targets

Targeting enzymatic co-factors of C-MYC-mediated transcription might circumvent the need to directly perturb C-MYC activity. Of these co-activators, CDK7 and CDK9 (as previously discussed), which mediate C-MYC expression as well as transcriptional activity, are being considered as therapeutic targets. Interestingly, relieving N-MYC-mediated gene repression using an HDAC inhibitor significantly curbed tumour development in an N-MYC-driven neuroblastoma mouse model (Liu et al., 2007). HAT inhibitors are in development (Bowers et al., 2010; Zheng et al., 2005), although further studies are required to determine their efficacy in vivo and in the context of C-MYC-deregulation. Moreover, as previously mentioned, inhibition of the C-MYC effector RNA pol I using a small molecule inhibitor specifically induced cell death in C-MYC-driven B cell lymphoma models (Bywater et al., 2012; Devlin et al., 2016). Knowing more about the relative importance of different co-activators

in C-MYC-mediated transcription will help elucidate which ones should be considered as potential therapeutic targets.

1.5.6 Synthetic lethal approaches to target C-MYC-dependent cells

Synthetic lethality, by definition, occurs when the perturbation of two particular genes or pathways manifests in cell death (or in the case of synthetic sickness – a less viable phenotype), whereas an individual one of said perturbations is tolerated by the cell. Identifying synthetic lethal interactions using knockout yeast strains has been a useful tool in identifying novel genetic pathways and novel genes within specific pathways (Forsburg, 2001). More recently, this approach has been utilised to identify synthetic lethal interactions with particular 'undruggable' oncogenes in human cells with a view to identifying novel targets for chemotherapies which would leave normal cells (without alteration of the particular oncogene) unscathed (Chan and Giaccia, 2011; Nijman, 2011). Logically, targeting these genes could be an effective, selective way to intervene with C-MYC driven cancers, particularly since targeting C-MYC directly and otherwise has yielded limited success thus far. High throughput screens and functional studies have identified synthetic lethal interactions with deregulated C-MYC in multiple pathways, interestingly some of which are not oncogenic drivers in themselves but are required for cell survival specifically upon C-MYC-mediated transformation.

Genes synthetic lethal with deregulated C-MYC include those involved in transcription initiation and elongation, C-MYC co-factors, translation initiation, metabolism and those involved in ubiquitin- and SUMO- regulation of DNA repair. For example, activation of C-MYC induces cellular dependency on ARK5

(5' adenosine monophosphate-activated protein kinase-related kinase 5) (Liu et al., 2012). ARK5 optimises the expression of mitochondrial respiratory chain components, and is thus required to metabolise glutamine for a source of cellular ATP. Cells with upregulated C-MYC exhibit enhanced glutamine metabolism, which is consistent with cellular addiction to ARK5.

Inhibition of the core spliceosome machinery also induces apoptosis specifically in C-MYC-driven-breast cancer cells (Hsu et al., 2015). Spliceosome perturbation caused intron retention to occur more frequently in cells with hyperactivated C-MYC compared to cells with basal C-MYC, likely because of the enhanced transcriptional load. The unspliced genes were shown to be involved in diverse essential cellular processes, explaining why cell viability was compromised.

C-MYC also exhibits synthetic lethal interactions with components of the eIF4F complex, which are also C-MYC target genes. eIF4F inhibition reduced proliferation of and induced apoptosis in C-MYC-driven premalignant pre-B/B-cells, while exhibiting minimal/reversible effects on normal cells (Lin et al., 2012a). In the presence of overexpressed C-MYC, eIF4F component expression was induced, and eIF4F inhibition was shown to deplete the expression of specific growth-promoting and anti-apoptotic genes. Interestingly, the same genes in wild-type cells with basal C-MYC levels were not affected by eIF4F inhibition.

It should be noted that a common method of C-MYC synthetic lethal interaction identification involves using RNA interference (RNAi) screens or focussed small molecule libraries. Each of these methods has its caveats: RNAi

libraries can cause variable protein depletion between genes, and small molecule libraries are limited to existing compounds. Therefore, there are likely unexplored synthetic lethal interactions with deregulated C-MYC which could be exploited as potential therapeutic targets.

1.6 Summary and aims

1.6.1 Summary

The transcription factor C-MYC is a potent oncogene frequently overexpressed or hyperactivated in cancers. Despite much effort to directly target C-MYC using small molecule compounds, no C-MYC inhibitors have been approved for cancer treatment. How C-MYC achieves its broad and varied effects on gene expression and cell physiology is still somewhat elusive, therefore understanding the intricacies of C-MYC function may illuminate a novel approach for cancer intervention. C-MYC regulates RNMT-RAM thus increasing mRNA cap methylation, which is important for C-MYC-driven protein synthesis. However, whether C-MYC also regulates formation of the guanosine mRNA cap is not known. Here, the mechanisms of C-MYC-mediated mRNA capping will be further investigated with an emphasis on the involvement of the enzyme which initiates mRNA cap formation - mRNA capping enzyme/CE.

1.6.2 Aims

- Determine if C-MYC regulates CE.
- Investigate whether CE is required for C-MYC function.

Chapter 2 : Methods and materials

2.1 Peptide pulldown

The protocol was adapted from a previous study (Ho and Shuman, 1999). Prior to incubation, magnetic streptavidin beads (Dynabeads M-280 streptavidin, Thermo Fisher) were washed three times in phosphate buffered saline (PBS) pH 7.4. All wash steps were performed using a magnetic rack (Dynal). Biotinylated CTD_3 and $(S5pCTD)_3$ peptides (Pepceuticals) were pre-bound to beads with rotation for 45 minutes at 4°C. 0.3-1nmol peptide and 15-25µl bead suspension were used per pulldown in 300µl binding buffer (Table 2.1) supplemented with 1mM dithiothreitol (DTT). Bead-peptide complexes were then washed three times with binding buffer. 1.2-4µg of recombinant protein was added to each pulldown in 50µl binding buffer and incubated with rotation at 4°C for 45 minutes. Supernatant was obtained (if analysing unbound fraction) and pulldowns were washed three times in binding buffer. Beads were eluted in F buffer with 1x Laemmli buffer and 0.1M DTT. 20% of eluate was analysed by SDS-PAGE and Western blotting.

2.2 Cell culture

All cells were maintained in 10cm plates at 37°C in a humidified incubator with 5% CO_2. Appropriate cell medium is indicated in Table 2.2. When cells reached approximately 80% confluency, cells were washed in PBS before 1ml of 0.05% trypsin-EDTA (Gibco) was added for 3-5 minutes. HeLa cells or PhoeNX cells were then resuspended in the appropriate culture medium before a fifth of the cell suspension was seeded on to a new plate. IMECs were

Buffer/solution	Composition
Binding buffer	25mM Tris/HCl pH 8, 50mM NaCl, 5% glycerol, 0.03% Triton X-100
F buffer	10mM Tris/HCl pH 7.05, 50mM NaCl, 30mM Na_4 pyrophosphate, 50mM NaF, 5µM $ZnCl_2$, 10% glycerol, 0.5% Triton x-100
Laemmli sample buffer (4x)	240mM Tris/HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue
Buffer A	10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl
Stacking gel mix	400mM Tris pH 6.8, 0.1% SDS, 0.1% APS, 4-5% acrylamide
Resolving gel mix	400mM Tris pH 8.8, 0.1% SDS, 0.1% APS, 5-12% acrylamide
SDS running buffer	25mM Tris, 250mM glycine, 0.1% SDS
Transfer buffer	25mM Tris, 192mM glycine, 20% methanol
TBS-tween (pH 8.1)	25mM Tris, 155mM NaCl, 0.1% Tween-20
Wash buffer	10mM Tris/HCl pH 7.5, 150mM NaCl, 0.5mM EDTA
Reaction buffer	50mM Tris/HCl pH 7.8, 5mM $MgCl_2$, 5mM DTT
High salt buffer	PBS, 0.5% NP-40, 1M NaCl
Low salt buffer	10mM Tris/HCl pH 8.0, 1mM EDTA, 0.1M NaCl, 0.5% NP-40
Sucrose cushion	Low salt buffer/100mM sucrose
NET buffer (11x)	550mM Tris/HCl pH 7.4, 1.56M NaCl, 5.5mM EDTA, 5.5% NP-40
RIPA buffer	50mM Tris/HCl pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40
LiCl buffer	10mM Tris/HCl pH 8.0, 250mM LiCl, 1mM EDTA, 0.5% deoxycholate, 0.5% NP-40
TE buffer	10mM Tris/HCl pH 8.0, 1mM EDTA

Table 2.1: Buffers and solutions.

The composition of buffers/solutions used in this thesis are indicated. Note that some of these require supplementation and this is described in the methods.

Cell line	Medium
HeLa	DMEM + 10% FBS, 2mM L-glutamine, 50 units/ml penicillin, 50µg/ml streptomycin
IMEC	DMEM/F12 (1:1) + 2mM L-glutamine, 5mg/ml insulin, 10ng/ml EGF, 0.5µg/ml hydrocortisone, 50 units/ml penicillin, 50µg/ml streptomycin, (±5% FBS)
PhoeNX	DMEM + 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 units/ml penicillin, 50µg/ml streptomycin
All (freezing medium)	70% FBS, 20% culture medium (see above), 20% DMSO

Table 2.2: Cell culture media.

Composition of culture medium is indicated. Note that for specific experiments, IMEC medium was supplemented with 5% FBS (see figure legends). DMEM/F12 medium was either obtained ready-made (Sigma-Aldrich or Gibco) or made by mixing DMEM 1:1 with F12. DMEM (Gibco); F12, Ham's F12 nutrient mixture (Gibco); FBS (Gibco); L-glutamine (Gibco); penicillin/streptomycin mix (Gibco); insulin, recombinant human insulin (Sigma-Aldrich); EGF, recombinant human epidermal growth factor (Sigma-Aldrich); hydrocortisone (Sigma-Aldrich); sodium pyruvate (Gibco); DMSO (Sigma-Aldrich).

resuspended in DMEM/10% FBS medium to neutralise trypsin before being centrifuged at 1,600rpm for 3 minutes. The pellet was resuspended in the appropriate growth medium and a third (IMEC/vec) or a fifth (IMEC/C-MYC) of cell suspension was seeded on to a new plate. Approximately every two months, cells in passage were replaced with a new frozen vial of cells.

2.3 Retroviral infection

IMECs were stably transduced with recombinant DNA vectors by using retroviral infection. A 10cm plate of PhoeNX cells was transfected with 4ug of DNA by using 8ug of polyethylenimine. The media was changed after 24 hours and, after a further 24 hours, media was removed from cells before being passed through a 0.45μm filter. The filtered media/viral supernatant was mixed 1:1 with recipient cell media and with 5μg/ml polybrene before being added to recipient cells. After 24-72 hours cells were selected with 150μg/ml hygromycin B (Sigma-Aldrich) for LxSH vectors (C-MYC constructs) or 500μg/ml G418 (Formedium) (for pBMN-IRES-Neo vectors (CE-GFP/CE-GFP WBL constructs).

2.4 Cell counting

Cells were counted by mixing cell suspension in growth media 1:1 with trypan blue (0.4%, Gibco) and then using a Countess cell counter (Life technologies) according to the manufacturer's instructions.

2.5 Cell cryostorage

Cells were trypsinised as before, except 0.5ml trypsin was used per 10cm plate. After 5 minutes, each plate of cells was resuspended in 1.5ml of

freezing medium (Table 2.2) and each 2ml was transferred to a cryovial. For long term storage, cells were frozen gradually via propanol submersion in a Mr Frosty container. After 1-3 days, cells were transferred to liquid nitrogen storage. For short term storage (<1 year), cells were stored at -80°C. To thaw cells, cryovials were submerged in a 37°C water bath and washed in culture media before being seeded on a 10cm plate.

2.6 Protein extraction

The entire procedure was performed on ice/at 4°C. Immediately before starting, F buffer was further supplemented with 1mM DTT, 1μM pepstatin, 10μM leupeptin, 0.1 trypsin inhibitor units aprotinin, 1% phosphatase inhibitor cocktail 2 and 1% phosphatase inhibitor cocktail 3 (all Sigma-Aldrich). Medium was removed from cells which were then washed twice in cold PBS. Supplemented F buffer was added to cells which were then removed from plates by scraping. The soluble fraction was collected from cell lysates after being centrifuged at 13,000rpm for 10 minutes. Protein concentration was determined by Bradford assay.

2.7 Nuclear protein extraction

The entire procedure was performed on ice/at 4°C. F buffer was supplemented, media was removed from cells and cells washed as above. Buffer A was added to cells which were then removed from plates by scraping. Cells were syringed 5 times with a 27G needle. Nuclei isolation was confirmed by mixing 5μl of sample 1:1 with 0.4% trypan blue and observing nuclear

staining using a microscope. Nuclei were pelleted by centrifugation at 6000rpm for 10 minutes before being lysed in F buffer as above.

2.8 Protein concentration determination

Bradford assay was used to determine protein concentration. Serial dilutions of bovine serum albumin (BSA) standard (Thermo Fisher) in F buffer were performed from 0.4-1.5mg/ml in 0.1mg/ml increments, plus 0 (blank) and 2mg/ml BSA, to generate a standard curve. 2µl of BSA dilutions were mixed with 200µl of 1x Bradford protein assay reagent (Bio-Rad) in a 96 well plate before absorbance was measured at 595nm using a plate reader. The blank reading was subtracted from BSA measurements. Absorbance was plotted against BSA concentration to derive a linear equation of y=0.196x, which was subsequently used to determine sample protein concentration. 1µl of each undiluted protein sample was first added to 1x Bradford reagent as above, and the colour shift was used as an approximation of how much each sample should be diluted. Samples were diluted in F buffer to similar protein concentrations within the linear range, before 2µl of diluted protein sample was added to 1x Bradford reagent and absorbance was measured as above. Each sample measurement was performed in triplicate wells.

2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking and resolving gel mix composition is indicated in Table 2.1. Polyacrylamide gel polymerisation was initiated by addition of 0.01% tetramethylethylenediamine (TEMED). Gels were resolved using mini-

PROTEAN tetra cells (Bio-Rad) in SDS running buffer according to the manufacturer's instructions. RNA pol II and SPT5 were analysed on resolving gels containing 5% acrylamide (4% stacking gel); p21 and p27 were analysed on gels containing 12% acrylamide (5% stacking gel); and all other proteins were analysed on gels containing 8% acrylamide (5% stacking gel). In certain experiments (indicated in figure legends), samples were resolved using a pre-cast NuPAGE 3-8% Tris-acetate gel (Thermo Fisher). In which case, gels were resolved using an X-cell SureLock mini-cell (Life Technologies) in 1x NuPAGE Tris-acetate SDS running buffer (Thermo Fisher) according to the manufacturer's instructions.

Samples were prepared for SDS-PAGE by using F buffer supplemented with Laemmli buffer and 0.1M DTT, diluting protein extracts to 1mg/ml. Samples were then boiled for 3-5 minutes before loading on a gel. Typically, 10-20µg of protein was loaded per well. 5µl of pre-stained protein ladder (PageRuler Plus, Thermo Fisher) was loaded for a molecular weight reference. 150-180V was applied until samples were resolved.

2.10 Western blotting

Following SDS-PAGE, proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane using the mini-trans-blot cell (Bio-Rad) in transfer buffer, according to the manufacturer's instructions. To prepare the 'sandwich', PVDF membranes were hydrated in methanol before being placed on the gel. This was placed between two 3mm Whattman filters followed by two Western blotting sponges; all pre-submerged in transfer buffer. 60-70V was applied to transfer proteins. For RNA pol II analysis, transfer was

performed for 2.5-3.0 hours due to the large molecular weight (>250kDa). For analysis of other proteins, transfer was performed for 1.5-3.0 hours. Ice packs were placed in transfer tanks, or tanks were placed in an ice bucket, to prevent over-heating.

Membranes were then blocked using TBS-tween supplemented with 5% milk (Marvel) or 3% BSA Fraction V (VWR). Primary antibodies are listed in Table 2.3, and were diluted in the appropriate blocking solution. Generally, membranes were incubated with primary antibodies for 2-2.5 hours at room temperature, although for analysis of PARP an overnight incubation at 4°C was required. Membranes were washed three times in TBS-tween/5% milk for a total of 45-60 minutes before being incubated with the appropriate horse radish peroxidase (HRP) -conjugated secondary antibody diluted in TBS-tween/5% milk for a further 45-60 minutes. Membranes were washed three times in TBS-tween for a total of 30-45 minutes before being incubated with SuperSignal West pico chemiluminescent substrate (Thermo Fisher) for 2 minutes according to manufacturer's instructions. Finally, membranes were exposed to X-ray films for various lengths of time before being developed in a film processor. When appropriate, Western blots were quantified using ImageJ software as indicated in figure legends.

2.11 Immunoprecipitation

Immunoprecipitations (IPs) were performed at 4°C with rotation and in F buffer supplemented as above. CE-GFP co-IPs were performed for 2.5 hours using GFP-Trap_A (GFP antibody conjugated to agarose beads, Chromotek). Other IPs were pre-cleared with 20ul of washed protein G agarose bead slurry

Target	Species/type	Company	Catalogue number	WB dilution	Blocking agent
CE/RNGTT	Sheep polyclonal	DSTT	n/a	1:750-1000	BSA
GST	Sheep polyclonal	DSTT	n/a	1:1000	Milk
S5p RNA pol II	Rat monoclonal	Chromotek	3E8	1:20	BSA
S2p RNA pol II	Rat monoclonal	Chromotek	3E10	1:15-20	BSA
RNA pol II pan (RPB1)	Rabbit polyclonal	Santa Cruz	sc-899	1:250-750	BSA
SPT5	Rabbit polyclonal	Cell Signalling	9033	1:500	BSA
C-MYC	Rabbit polyclonal	Cell Signalling	9402	1:750-1000	BSA
PARP	Rabbit polyclonal	Cell Signalling	9542	1:1000	BSA
p21	Rabbit polyclonal	Santa Cruz	sc-397	1:1000	BSA
p27	Rabbit monoclonal	Abcam	ab32034	1:1000	BSA
Actin	Mouse monoclonal	Abcam	ab3280	1:2000	Milk
Tubulin	Rabbit polyclonal	Santa Cruz	sc-9104	1:2000	Milk
SMC1	Rabbit polyclonal	Bethyl laboratories	A300-055A	1:2000	BSA
Nucleolin/NCL	Rabbit polyclonal	Bethyl laboratories	A300-711A	1:1000	BSA
GFP	Mouse, mix of two monoclonals	Roche	11814460001	1:1000	BSA
N-MYC	Rabbit polyclonal	Cell Signalling	9405	1:250	BSA
HIF1α	Rabbit polyclonal	Santa Cruz	sc-10790	n/a	n/a
FLAG (M2)	Mouse monoclonal	Sigma	F1804	n/a	n/a

Table 2.3: Antibodies used for Western blotting and IP.

The antibody species, type, provider and conditions for Western blotting are indicated. Amount of antibody used for IP is indicated in figure legends. n/a, not applicable.

(Generon) for 1 hour before the antibody was added, and another 20ul of washed bead slurry added for 1 hour to bind the antibody. IPs were washed three times in wash buffer and then eluted in F buffer with 1x Laemmli buffer and 0.1M DTT. Samples were analysed by SDS-PAGE followed by either Western blotting or autoradiography. Amount of antibody and protein in IP, length of IP, and amount of eluate analysed is indicated in figure legends. Antibodies used for IP are included in Table 2.3. To perform co-IPs using HeLa cells, $1.5x10^6$ cells were seeded on 10cm plates the day before lysis. To perform co-IPs using IMECs (including for guanylyltransferase assays), $2.3x10^6$ cells were seeded on 15cm plates 24 hours prior to lysis. To perform CE IPs for guanylyltransferase assays, $1x10^6$ IMECs were seeded 24 hours prior to lysis on 10cm plates.

2.12 Guanylyltransferase activity assay

The guanylyltransferase activity assays were adapted from previous publications (Ho and Shuman, 1999; Ho et al., 1998). Cell lysis and IPs were performed as described above, except F buffer was supplemented with 5mM DTT. After IP, samples were washed in F buffer before being incubated with 3.75U/ml RNase A and 150 U/ml RNase T1 (RNase cocktail enzyme mix, Thermo Fisher) in F buffer at room temperature for 15 minutes. IPs were then washed three times in reaction buffer before being incubated for 1 minute at 37°C in reaction buffer/6.7μM [α-^{32}P]GTP (BLU006H500UC, Perkin Elmer). IPs were eluted as above and analysed by SDS-PAGE. Gels were then dried and formation of CE-[α-^{32}P]GMP covalent intermediate was quantified with a phosphorimager (FUJIX BAS2500) and AIDA image analyser.

2.13 siRNA transfection

Small interfering RNA (siRNA) transfections were performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. Immediately after seeding cells, transfection mix (containing 1:1:50 volumetric ratio of transfection reagent, 50μM siRNA and DMEM) was added to culture medium to give a final siRNA concentration of 50nM for single target knockdowns or 75nM for double target knockdowns. Final concentration of siRNA and length of knockdown is specified in figure legends. siRNA target sequences are depicted in Table 2.4. For transfections prior to CE ChIP, $1.5x10^6$ cells were seeded on 10cm plates. For transfections prior to RNA pol II ChIP, $1.0x10^6$ cells were seeded on 10cm plates. For transfections followed by qRT-PCR, Western blot analysis or cell counting (after 72 hours), $1.1x10^5$ or $2.3x10^5$ cells. were seeded on 6 well plates or 6cm plates, respectively. For knockdowns longer than 24 hours, culture medium was changed at this time.

2.14 RNA extraction

RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA pellets were resuspended in 20-50μl of Milli-Q water and RNA concentration was measured with a NanoDrop 2000 spectrophotometer.

2.15 Complementary DNA (cDNA) synthesis

cDNA synthesis reactions (20μl) containing 1μl reverse transcriptase (RT) and 200ng of RNA were prepared using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

Target	Cat. no.	Target sequence
C-MYC	D-003282-14-0050	AACGUUAGCUUCACCAACA
CE (siRNA #1)	D-009782-01-0050	CAAAAGAGCUGAAACAGUA
CE (siRNA #2)	D-009782-02-0050	CUAAAGAGCCUAAAGGUUA
Non-targeting control	D-001210-03-50	-

Table 2.4: siRNA oligos for cell culture transfection.

The corresponding mRNA target sequence for each siRNA used in this thesis is indicated. siRNAs were purchased from Dharmacon as lyophilised powder and were resuspended in sterile PBS to a stock concentration of 50μM.

2.16 Quantitative polymerase chain reaction (qPCR)

qPCRs were performed with SsoFast EvaGreen supermix (Bio-Rad) according to manufacturer's instructions. Primers for qRT-PCR and ChIP qPCR are indicated in Tables 2.5 and 2.6, respectively. 5µl reactions were performed using 1µl cDNA, and 10µl reactions were performed using 1µl ChIP DNA; both in 384 well plates.

2.17 Chromatin immunoprecipitation (ChIP)

The protocol was adapted from a previous study (Varshney et al., 2015). One or two plates of HeLa cells were used for each RNA pol II ChIP or CE ChIP, respectively. siRNA transfection conditions prior to ChIP are specified in section 2.13 and in figure legends. Protein–DNA complexes were cross-linked using 1% formaldehyde (Thermo Fisher) in growth media for 10 minutes (RNA pol II ChIP) or 15 minutes (CE ChIP) with gentle agitation at room temperature. The reaction was quenched by adding 0.125M glycine and incubating with gentle agitation at room temperature for 5 minutes. The rest of the protocol was performed on ice/at 4°C unless otherwise stated. Cells were removed from plates by scraping and washed twice in PBS then twice in PBS/0.5% NP-40. Nuclei were isolated by incubation in high salt buffer for 30 minutes followed by low salt buffer for 30 minutes before being cleaned by centrifugation through two sucrose cushions. The chromatin was then sheared by water bath sonication using a Bioruptor (Diagenode) at high intensity for 30 minutes of 30 second on/off cycles, yielding fragment sizes of 200-500bp. Samples were pre-cleared for 1 hour with 20µl slurry of washed Protein A agarose beads blocked with salmon sperm DNA (Millipore). 10% of each sample was removed for input

Target (cDNA)	Forward primer	Reverse primer
C-MYC	5'-TCTGAGGAGGAACAAGAA-3'	5'-GAAGGTGATCCAGACTCT-3'
FLAG-MYC (mouse)	5'-CACGGAGGAAAACGACAAGA-3'	5'-GAATGGACAGGATGTAGGCG-3'
NCL	5'-TGCCAGAAGCCAGCCATCCAAA-3'	5'-GCCCGAACGGAGCCGTCAAAT-3'
ODC	5'-CGCTGTGACCTGCCTGAAATG-3'	5'-TGCATGAGTTGCCACGCAGGC-3'
FBL	5'-AAGAATGTGATGGTGGAGCC-3'	5'-GTGACCAGTGCATCTTCCTT-3'
NME1	5'-TCATGCAAGCTTCCGAAGATC-3'	5'-GCCCTGAGTGCATGTATTTCAC-3'
NPM	5'-GAAGAGGAGGAGGATGTG-3'	5'-TTCTGTGGAACCTTGCTA-3'
TIP49	5'-CATTGGGCTGCGAATAAA-3'	5'-TCTGTCTCACACGGAGTT-3'
UBF	5'-CTGAGATGAGCAACCTGGAC-3'	5'-ACCTTCTTCTCGTGGGTGTA-3'
CCND1	5'-CATTGAACACTTCCTCTCC-3'	5'-ATGAACTTCACATCTGTGG-3'
GAPDH	5'-GGAGTCAACGGATTTGG-3'	5'-GTAGTTGAGGTCAATGAAGGG-3'
CE	5'-ATTGGAGTATCGAAGCAG-3'	5'-GGTGCTTCCTCTATGTCACC-3'
eIF4E	5'-AACGAATGACCACCAGCATT-3'	5'-GATGCAGAGTCGTTTAGGCA-3'
CRD-BP	5'-GTACCAAGAGACCAGACCCC-3'	5'-GATCTTCCGTTGAGCCATCT-3'

Table 2.5: RT-PCR primers.

The corresponding cDNA target sequence for each RT-PCR primer set used in this thesis is indicated. Oligos were purchased from Life Technologies.

Target (gDNA)	Forward primer	Reverse primer
NCL -2500	5'-GTTTGTAATGG CCAAACATAGCA-3'	5'-GGCATTCCTTGTGTGGAAGG-3'
NCL TSS	5'-TTTCCACAGGCGATTACTGG-3'	5'-GAGCACGTACACCCGAAG-3'
NCL 500	5'-TTTTGCGACGCGTACGAG-3'	5'-ACTAGGGCCGATACCGCC-3'
NCL 1000	5'-CGGTTGAGAGTAGTCCCTCT-3'	5'-TCATCTCCGTCCTCAGATCC-3'
FBL -200	5'-GGGAAGCCTTTTTGGGTGTA-3'	5'-CACCCCAATAATCAGGGCTC-3'
FBL TSS	5'-GAGAATCCAGGCTCACTGC-3'	5'-GTTCACAACTCCACGAGTCC-3'
FBL 300	5'-GCCATGAAGCCAGGTCAG-3'	5'-CTTCCCACAGGAGACTGGAA-3'
NME1 TSS	5'-GAATGACTGCC TACTCCCAAGAG-3'	5'-CACGCACGGAACGCTTCT-3'
NME1 500	5'-CCTTTCTCGTCAGGCCG-3'	5'-CTTACAGAGCGCCAACTCC-3'
GAPDH TSS	5'-TACTAGCGGTTTTACGGGC-3'	5'-GCTGCGGGCTCAATTTATAG-3'
C-MYC -2000	5'-AAGACGCTTTGCAGCAAAATC-3'	5'-AGGCCTTTGCCGCAAAC-3'
C-MYC TSS	5'-GGAGGGATCGCGCTGAGT-3'	5'-TATTCGCTCCGGATCTCCCT-3'
C-MYC 450	5′-GCACTGGAACTTACAACACC-3′	5′-ATCCAGCGTCTAAGCAGC-3′
C-MYC 3600	5‘-GTCCAAAGCCTCA TTAAGTCTTAGGTA-3'	5'-CAACTTCCCAG GATAGGACATTG-3'
TIP49 300	5′-TGTGGCCAGTGGACC-3′	5′-ACTTCCCTGAGGAAATAATGG-3′
CCND1 250	5′-AGCTGCCCAGGAAGAGC-3′	5′-CCGCCTTCAGCATGG-3′
RNMT 100	5′-TGAGTGTGACGGCTGGAACTC-3′	5′-CACGCGTTGGGTAGTGAAG-3′

Table 2.6: ChIP primers.

The corresponding genomic DNA (gDNA) target sequence for each ChIP primer set used in this thesis is indicated. Oligos were purchased from Life Technologies.

material to include in final qPCR analysis and ensure equal loading of chromatin into IPs. Immunoprecipitation was performed overnight in 1x NET buffer using 1µg of RNA pol II or CE antibody. 20µl bead slurry was then added to each sample for 1 hour. Immunoprecipitates were washed twice in RIPA buffer, twice in LiCl buffer and twice in TE buffer before being eluted in TE/1% SDS for 30 minutes with rotation at room temperature. 125µg/ml proteinase K was added to samples and incubated overnight at 42°C. DNA was purified with QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 50µl Milli-Q water and analysed by qPCR. Input samples were diluted 10-fold for PCR reactions.

2.18 Anchorage-independent cell growth assay

$0.75x10^5$ IMECs (maintained in 5% FBS growth media for at least a week beforehand) were plated in 6 well plates and transfected with siRNA as above. After 72 hours, $0.8x10^4$ cells were plated in 2ml of growth medium/5% FBS containing 0.33% noble agar in a 6 well plate on top of a 0.6% noble agar base layer (2ml). Technical triplicates were performed for each condition within each biological replicate. Cells were fed with 500µl of growth media/5% FBS every second day. After 9-15 days, colonies were scored using a graticule. Total number of cells (undivided and divided), colonies >20µm and colonies >50µm were counted in five randomly selected fields of each well. Micrographs were taken after one month. Note that noble agar stock solutions (3.3% and 6%) were sterilised by autoclaving in advance.

2.19 Cell viability assay

CellTiter-Blue viability assays were performed according to manufacturer's instructions (Promega). Media was removed from cells growing in 96 well plates. 120µl of pre-warmed 1x CellTiter-Blue reagent in the appropriate medium was added to cells (or empty wells as blank measurements). Cells were incubated at 37°C for 2 hours before fluorescence was measured at 590nm using a Gemini XPS microplate reader. Cell number per well was optimised (Figure 5.7) and for each experiment cell number is indicated in figure legends.

2.20 Inhibitor treatment

Inhibitors used in this thesis – MG132 (Merck) and THZ1 (see below) – were dissolved in DMSO, therefore as a negative control cells were treated with an equal volume of DMSO alone. If two inhibitor concentrations were used, the DMSO control corresponded to the higher inhibitor concentration. In 96 well THZ1 titration assays, DMSO controls were performed for each THZ1 concentration, and each condition was performed in triplicate wells. Since THZ1 is a covalent CDK7 inhibitor, THZ1 and control treatment were washed out (i.e. media changed) after 4 hours to minimise off-target effects (Kwiatkowski et al., 2014). Treatments were performed the day after cell seeding (unless siRNA was also used; indicated in figure legends). Inhibitor concentration and length of treatments are specified in figure legends. THZ1 was a gift from Dr. Nathanael Gray (Dana Farber Cancer Institute, Boston) to Dr. Francisco Inesta-Vaquera in the Cowling laboratory.

2.21 Statistical analysis

Statistical significance was calculated by two-tailed Student's t-test (assuming equal variance) throughout this thesis. Paired t-tests were performed between conditions (for example CE siRNA versus non-targeting control) and unpaired t-tests were performed between stable cells lines (i.e. IMEC/vec and IMEC/C-MYC). Data from at least three independent experiments was used for statistical tests ($n \geq 3$). Sample size for each experiment is indicated in figure legends. For Kaplan-Meier curves, statistical significance was calculated using the log rank test, and sample size is indicated on graphs.

Chapter 3 : C-MYC regulates mRNA capping enzyme

3.1 Introduction

C-MYC increases the expression and recruitment of CDK7 and CDK9, leading to a global increase in S5 and S2 RNA pol II CTD phosphorylation (Cowling and Cole, 2007b). These modifications are essential for the association of a range of factors involved in transcription initiation, elongation, mRNA capping, splicing and transcription termination (Heidemann et al., 2013). C-MYC depends on the recruitment of these factors for driving expression of its target genes. Since attempts at therapeutically targeting C-MYC directly have been largely unfruitful, these components could provide an alternative route to block C-MYC function.

C-MYC regulates RNMT to catalyse formation of the 7-methylguanosine cap structure linked to the first transcribed nucleotide on its target transcripts, which contributes to C-MYC-dependent gene expression and cell proliferation (Cowling and Cole, 2007b; Fernandez-Sanchez et al., 2009). The mRNA cap protects transcripts from degradation, promotes splicing and 3' end processing, facilitates nuclear export of mRNA, and facilitates translation initiation. It has not yet been determined whether C-MYC also regulates other stages of mRNA cap formation.

The enzyme which initiates cap formation, mRNA capping enzyme/RNA guanylyltransferase and 5' triphosphatase (CE/RNGTT), specifically binds to the RNA pol II CTD when phosphorylated at S5, spatially and temporally localising the enzyme to cap nascent mRNA as it emerges from RNA pol II (Ghosh et al., 2011; Ho et al., 1998; Martinez-Rucobo et al., 2015; McCracken et al., 1997). In

vitro, CE also interacts with S2p RNA pol II CTD and a mediator of RNA pol II elongation – the SPT5 subunit of DSIF – but the mechanism of binding and biological significance of these interactions are not well understood (Ho and Shuman, 1999; Mandal et al., 2004; Wen and Shatkin, 1999). CE has triphosphatase and guanylyltransferase activities which act sequentially to catalyse addition of the basic guanosine cap structure. S5p RNA pol II CTD and SPT5 per se stimulate CE guanylyltransferase activity in vitro (in contrast to S2p RNA pol II CTD which binds CE but does not alter activity) (Ho and Shuman, 1999; Mandal et al., 2004; Wen and Shatkin, 1999). RNMT methylates the guanosine cap moiety in complex with its activating subunit RAM to complete the mRNA cap structure. Although C-MYC regulates RNMT-RAM, whether CE is also regulated to achieve C-MYC-dependent mRNA capping is not clear.

In this chapter, it was investigated whether C-MYC modulates recruitment of the mRNA capping machinery to transcription complexes.

3.2 Results

3.2.1 C-MYC increases the interaction of CE with RNA pol II and SPT5

To investigate the interaction of capping enzymes with the RNA pol II CTD in vitro, GST-tagged recombinant CE and RNMT were incubated with RNA pol II CTD peptides. Biotinylated peptides were used which consisted of three unphosphorylated heptad repeats – $(YSPTSPS)_3$ / CTD_3 – or three repeats phosphorylated at S5 residues – $(YSPTSpPS)_3$ / $(S5pCTD)_3$. As previously observed, CE bound specifically to $(S5pCTD)_3$ peptides and not to CTD_3 peptides (Figure 3.1 A) (Ho and Shuman, 1999). In a preliminary experiment, RNMT and RAM did not interact with the $(S5pCTD)_3$ or CTD_3 peptides (Figure 3.1 B), consistent with previous observations. Since CE specifically binds phosphorylated RNA pol II, its recruitment and activity has the potential to be regulated by C-MYC.

To further investigate the interaction of CE and RNA pol II, co-immunoprecipitations were performed with HeLa cell protein extracts (Figure 3.2). Interestingly, only a small proportion of total S5p RNA pol II co-purified with CE. The interaction of CE with SPT5 was also investigated, and an even smaller proportion of SPT5 co-purified with CE relative to S5p RNA pol II. However, RNA pol II interacts with SPT5 and therefore it is not clear whether the interaction between CE and SPT5 is direct. RNA pol II is one of the top interactors of CE as demonstrated by CE-GFP immunoprecipitation and mass spectrometry (unpublished data, Dr D. Varshney/Cowling lab), indicating that this is an important interaction. It should be noted that control IPs should be

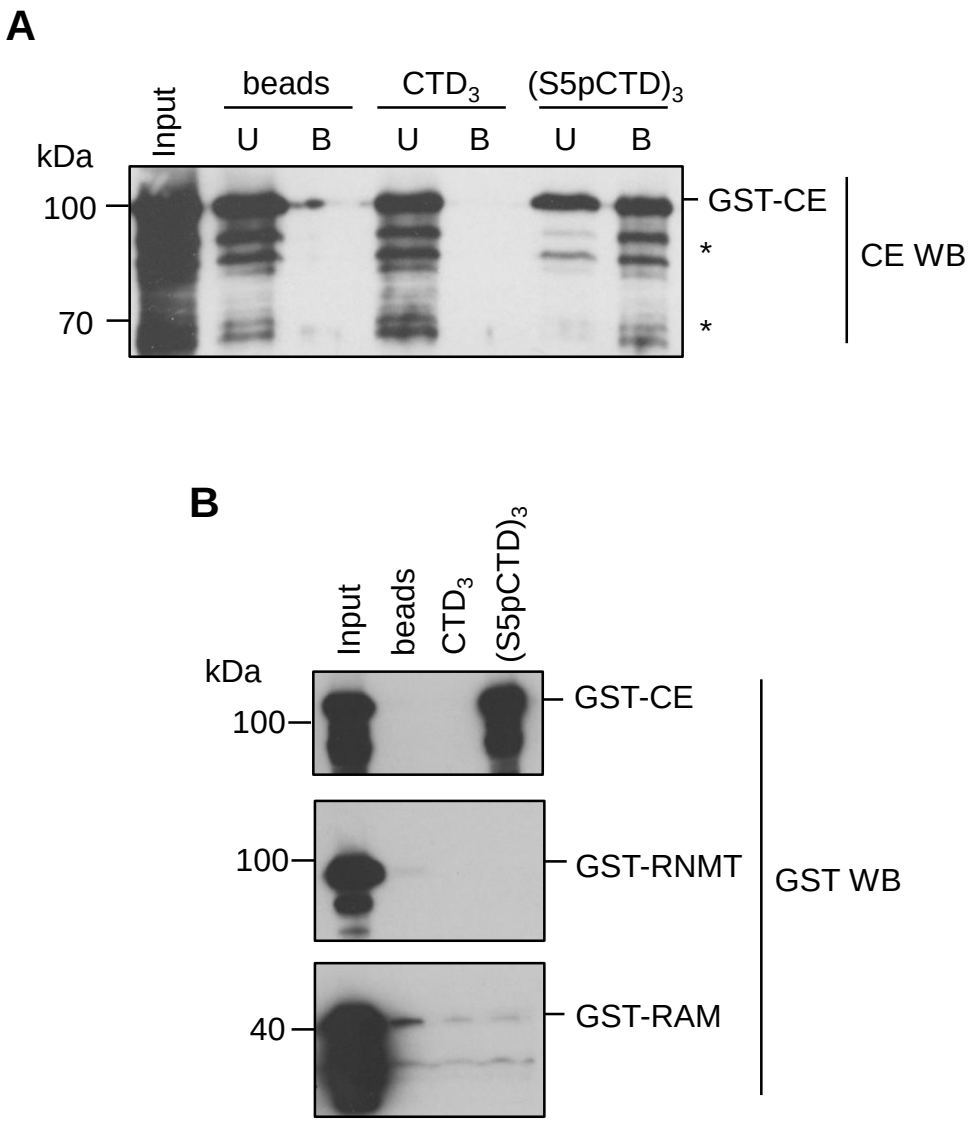

Figure 3.1: CE binds S5p RNA pol II CTD in vitro. (A) Recombinant GST-tagged CE was incubated with streptavidin beads alone or with immobilised biotinylated peptides; either three tandem unphosphorylated CTD repeats - CTD_3 - or three tandem S5 phosphorylated CTD repeats - $(S5pCTD)_3$.Bound (B) and unbound (U) fractions were analysed by SDS-PAGE and Western blotting using a CE antibody. *potential breakdown products. Representative of two independent experiments. (B) Recombinant GST-tagged CE, RNMT and RAM were incubated with beads alone or with immobilised peptides on beads as above. Bound fractions were analysed by SDS-PAGE and Western blotting using a GST tag antibody. Individual experiment.

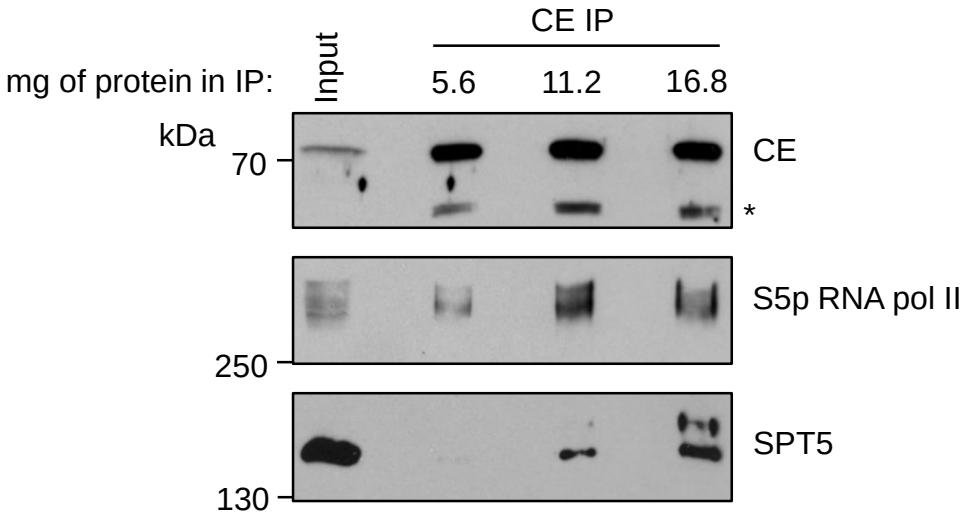

Figure 3.2: A small fraction of total cellular CE co-purifies with RNA pol II and SPT5. CE was immunoprecipitated from HeLa cell protein extracts. Three distinct IPs were performed overnight with 5.6mg, 11.2mg or 16.8mg of protein and 1µg, 2µg or 3µg of CE antibody, respectively. Samples were analysed by SDS-PAGE and Western blotting. 80% of IP eluate was loaded for S5p pol II and SPT5 Western blots (same gel) and 3% of IP eluate was loaded for the CE Western blot. 20µg of input material was loaded for comparison. *non-specific/likely heavy chain. Individual experiment.

performed for the above experiment to ensure the co-purification of RNA pol II with CE is not due to non-specific binding to the antibody/beads.

In order to determine if the interaction of CE and RNA pol II was modulated by C-MYC, retroviral infection was used to stably overexpress *C-MYC* (Figure 3.3). Expressing wild type (WT) *C-MYC* did not increase total C-MYC protein levels compared to the empty vector (vec). This could be because C-MYC represses its own expression at a critical threshold as a negative-feedback mechanism (Cleveland et al., 1988; Penn et al., 1990). Overexpressing a mutant version of *C-MYC* (*C-MYC* T58A), which as previously discussed encodes a substantially more stable version of the protein (Sears et al., 2000), only slightly increased net C-MYC protein levels. The *C-MYC* gene in HeLa cells exhibits strong transcriptional activation due to viral insertion, thus these cell already have high levels of endogenous C-MYC (Adey et al., 2013). Although *C-MYC* T58A increased S5p RNA pol II levels (Figure 3.3), cells proliferated slower than cells expressing the empty vector control (data not shown). Perhaps ectopic C-MYC T58A in HeLa cells is toxic, since it is known that gross C-MYC activity in some systems can induce apoptosis as a tumour suppressor mechanism (Murphy et al., 2008). Therefore, this system was not optimal and a different system was adopted to overexpress C-MYC.

IMECs are a non-transformed human cell line which are immortalised by overexpression of the catalytic subunit of telomerase (hTERT) (DiRenzo et al., 2002). They exhibit growth control mechanisms such as cell cycle checkpoints, contact inhibition and anchorage dependency, and display genome integrity in cell culture (Cowling et al., 2007; Toouli et al., 2002). *C-MYC* is endogenously expressed in IMECs, but at low basal levels. Retroviral infection was used to

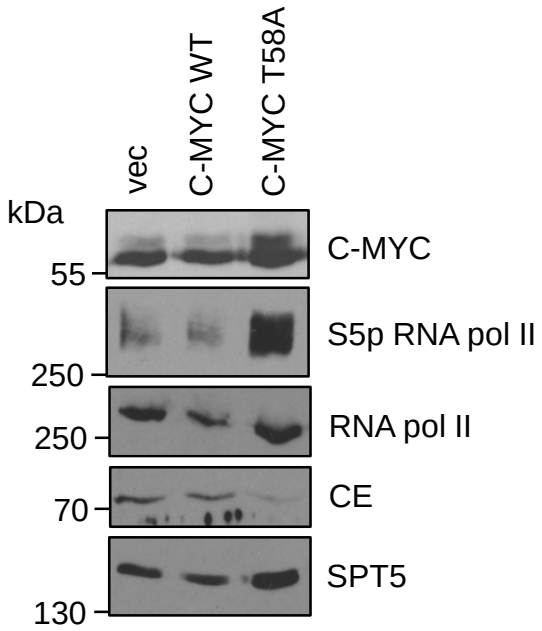

Figure 3.3: *C-MYC* overexpression in HeLa cells. HeLa cells were stably transduced with empty vector (vec), FLAG-tagged wild-type *C-MYC* vector (C-MYC WT) or a FLAG-tagged mutant *C-MYC* vector which encodes a stabilised version of the protein (C-MYC T58A). Protein extracts were analysed by SDS-PAGE and Western blotting. Representative of two independent experiments.

stably express empty vector or WT *C-MYC*, establishing IMEC/vec and IMEC/C-MYC cell lines, respectively. A second round of infection stably expressed CE-GFP or control (INI). Equivalent levels of CE-GFP were expressed in IMEC/vec and IMEC/C-MYC, and CE-GFP expression did not alter C-MYC expression (Figure 3.4 A). To determine if the interaction of CE-GFP with RNA pol II was C-MYC-responsive, CE-GFP was immunoprecipitated from IMEC/vec and IMEC/C-MYC protein extracts via the GFP tag (Figure 3.4 B). C-MYC increased the interaction of CE-GFP with RNA pol II. The vast majority of the co-purified RNA pol II was of a higher molecular weight compared to that in the input, indicating phosphorylated RNA pol II is interacting with CE-GFP in cells. However, IPs should be treated with phosphatase to confirm the phosphorylated status of RNA pol II. To observe this interaction with endogenous protein, CE was immunoprecipitated from IMEC/vec and IMEC/C-MYC protein extracts (Figure 3.5 A and B). As previously observed (Cowling and Cole, 2007b), C-MYC increased S5p RNA pol II levels. Overexpressing C-MYC increased co-purification of phosphorylated RNA pol II with endogenous CE. This was observed using both a phospho-specific antibody recognising S5p RNA pol II CTD and a pan RNA pol II antibody. Elevated C-MYC expression also increased the co-purification of SPT5 with CE. As previously mentioned, this could represent a direct interaction between CE and SPT5, or an indirect association via RNA pol II. Previous screens for C-MYC interacting partners (Agrawal et al., 2010; Dingar et al., 2015) and CE interacting partners (unpublished data, Dr D. Varshney/Cowling lab) have not identified CE and C-MYC in a complex, thus C-MYC-induced CE recruitment is likely not via a direct interaction between the two proteins. In summary, C-MYC promotes the recruitment of CE to RNA pol II and SPT5.

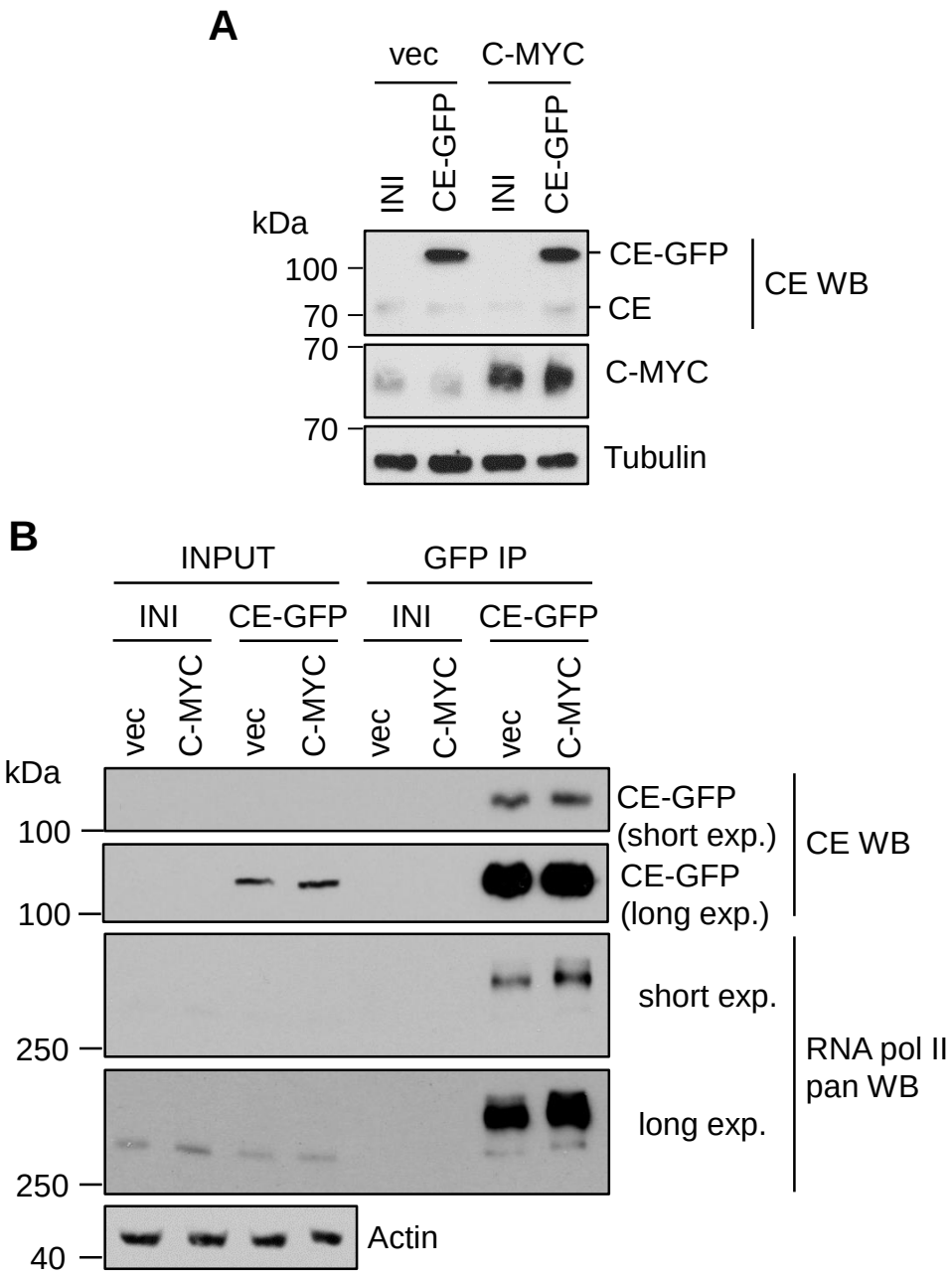

Figure 3.4: C-MYC overexpression in IMECs increases the interaction of CE-GFP with RNA pol II. (A) Protein extracts of IMEC/vec and IMEC/C-MYC stably transduced with CE-GFP or empty vector (INI) were analysed by Western blotting. Individual experiment. This was performed in collaboration with Dr. Dhaval Varshney in the Cowling lab. (B) CE-GFP was immunoprecipitated from protein extracts of IMEC/vec and IMEC/C-MYC expressing CE-GFP or empty vector for 2.5 hours using GFP antibody-conjugated beads. 9mg of protein was used in each IP. 80% of eluate was loaded for RNA pol II Western blots and 10% for CE Western blots. 15µg of input material was loaded for comparison. Representative of two independent experiments.

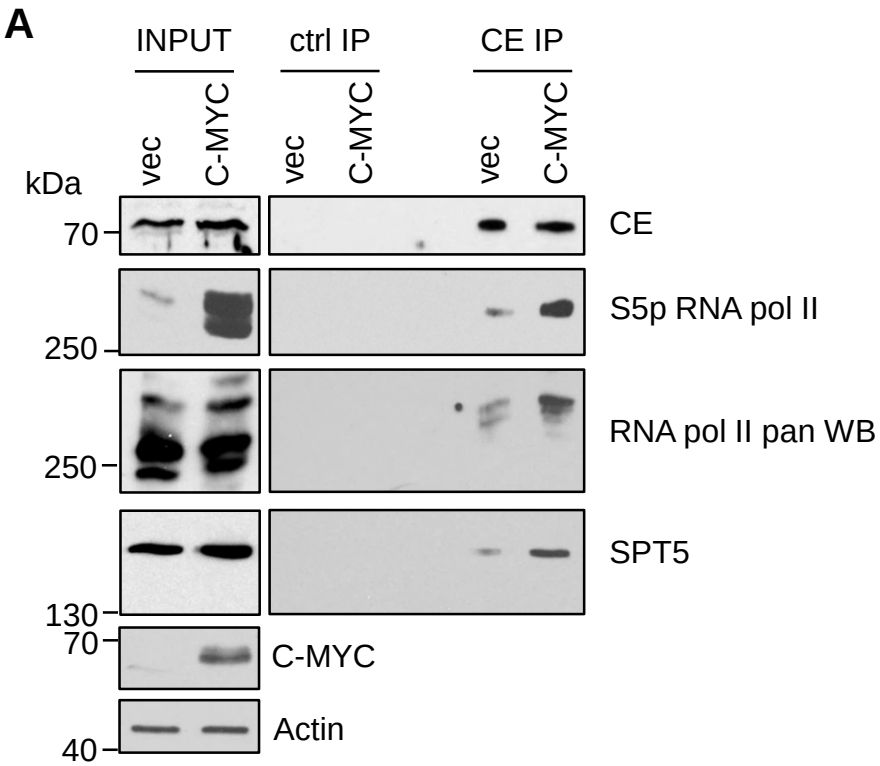

Figure 3.5: C-MYC overexpression increases the interaction of endogenous CE with S5p RNA pol II and SPT5. (A) CE immunoprecipitation was performed for 2.5 hours from IMEC/vec and IMEC/C-MYC protein extracts. IPs using a FLAG tag antibody were performed as a control (ctrl IP). 4.7mg protein and 2µg antibody was used in each IP. 40% of eluate was loaded for S5p RNA pol II, SPT5 and RNA pol II Western blots (the former two from the same gel) and 4% loaded for the CE Western blot. Input and IP panels are from the same Western blots but are different exposures. Representative of four (S5p RNA pol II) or two (RNA pol II pan and SPT5) experiments. Figure continued overleaf.

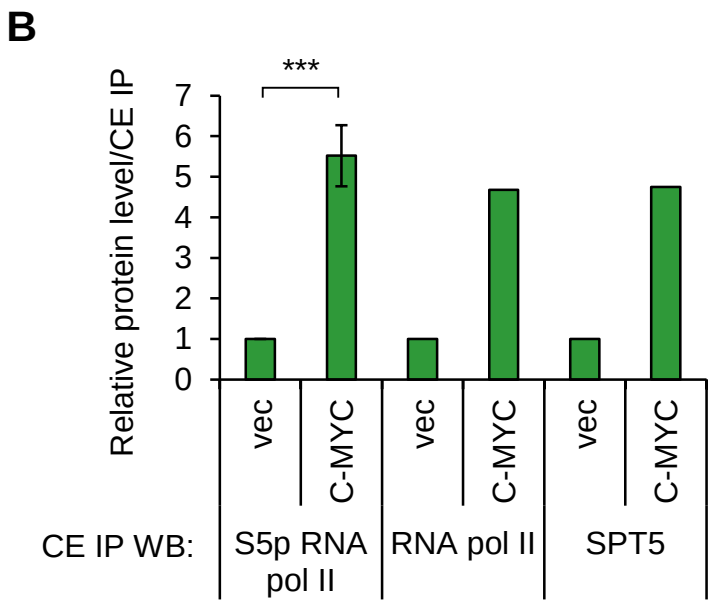

Figure 3.5 continued: C-MYC overexpression increases the interaction of endogenous CE with S5p RNA pol II and SPT5. (B) Densitometry was performed using ImageJ software to quantify the Western blot signal of S5p RNA pol II, RNA pol II pan and SPT5 following CE immunoprecipitation, normalised to the CE IP Western blot signal. Both bands in the RNA pol II pan WB are included in the quantification. Error bars represent standard error of the mean, n=4; RNA pol II pan and SPT5 WB signals are averaged from two independent experiments. Significance was calculated by Student's t-test; *** $p \leq 0.001$.

To confirm the relationship between C-MYC expression and CE recruitment, RNA pol II and SPT5 were immunoprecipitated from IMEC/vec and IMEC/C-MYC protein extracts to observe the reciprocal interaction (Figure 3.6 A and B). However, CE could not be detected in RNA pol II nor SPT5 IPs. This could be due to poor immunoprecipitation efficiency (as suggested by the amount of RNA pol II in the flow-through), or the fact that the RNA pol II antibody IPs the non-phosphorylated form of the protein more efficiently than the higher molecular weight RNA pol II (compare relative amounts in the IP with that in the input).

To further investigate C-MYC-dependent CE recruitment, an additional set of IMEC stable cell lines was made expressing C-MYC mutants (Figure 3.7 A). C-MYC ΔMBII and C-MYC T58A were expressed in addition to C-MYC WT. C-MYC ΔMBII lacks the MBII component of the TAD which is required for the interaction of C-MYC with TFIIH/CDK7 (and other transactivators) and is defective in stimulating RNA pol II S5 phosphorylation (Cowling and Cole, 2007b). As previously mentioned, C-MYC T58A is more stable and its expression results in elevated steady-state C-MYC levels (Figure 3.7 A). As expected, C-MYC ΔMBII did not increase S5p RNA pol II levels and C-MYC T58A caused a greater increase in S5p RNA pol II levels than C-MYC WT (Figure 3.7 A). Preliminary experiments showed that C-MYC ΔMBII was not able to increase the interaction of CE with RNA pol II (Figure 3.7 A and B), indicating that C-MYC-mediated CE recruitment is dependent on its ability to stimulate RNA pol II S5 phosphorylation. Moreover, C-MYC T58A had a heightened capability to induce the interaction of CE with S5p RNA pol II relative to C-MYC WT (Figure 3.7 A and B), demonstrating a dose-dependent

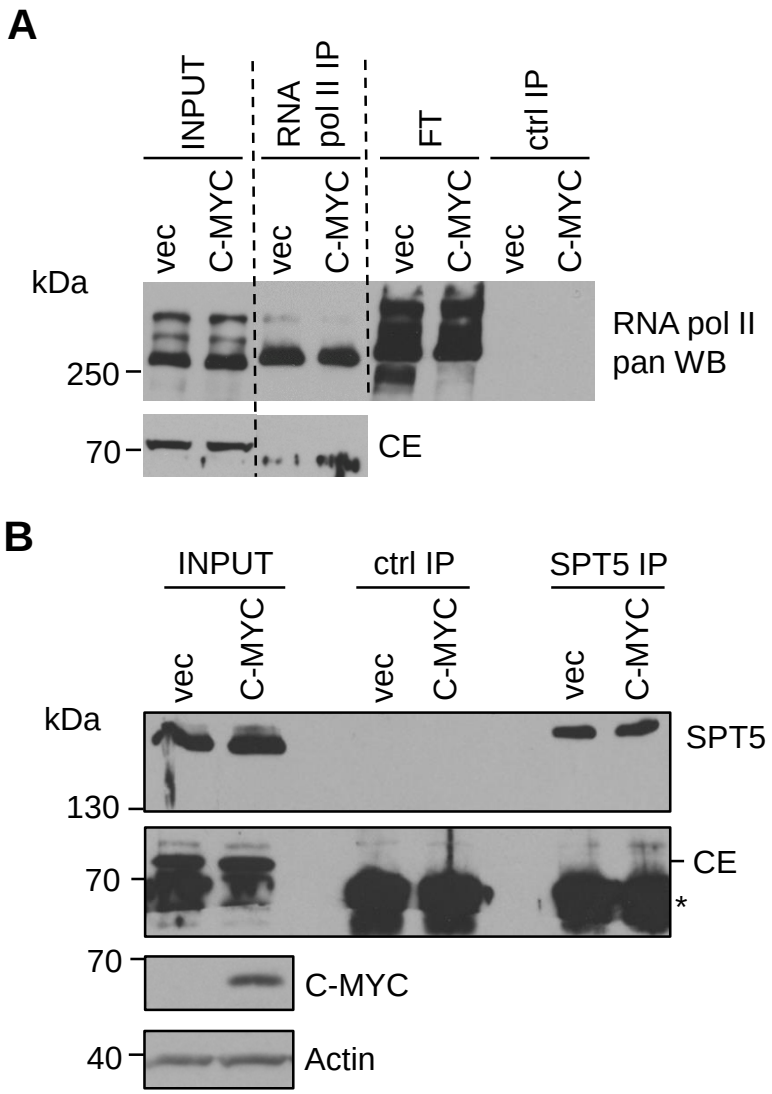

Figure 3.6: The reciprocal immunoprecipitation of CE with RNA pol II and SPT5 if not detectable. (A) RNA pol II was immunoprecipitated from 2.9mg IMEC/vec and IMEC/C-MYC protein extracts for 2.5 hours with 1µg antibody. IPs with a GST antibody were performed as a control (ctrl IP). 30% of eluate was loaded for CE and RNA pol II Western blots. ~5% of the unbound flow-through (FT) was loaded to examine RNA pol II IP efficiency. 20µg of input material was loaded for comparison. Samples are from the same Western blots and same exposures (indicated by dashed lines). Representative of three independent experiments. (B) SPT5 was immunoprecipitated from 8.9mg IMEC/vec and IMEC/C-MYC protein extracts for 2.5 hours with 5µl antibody. HIF1α IPs were performed as a control (ctrl IP). 80% of eluate was loaded for the CE Western blot and 10% for the SPT5 Western blot. 20µg of input material was loaded for comparison. *non-specific band(s). Individual experiment.

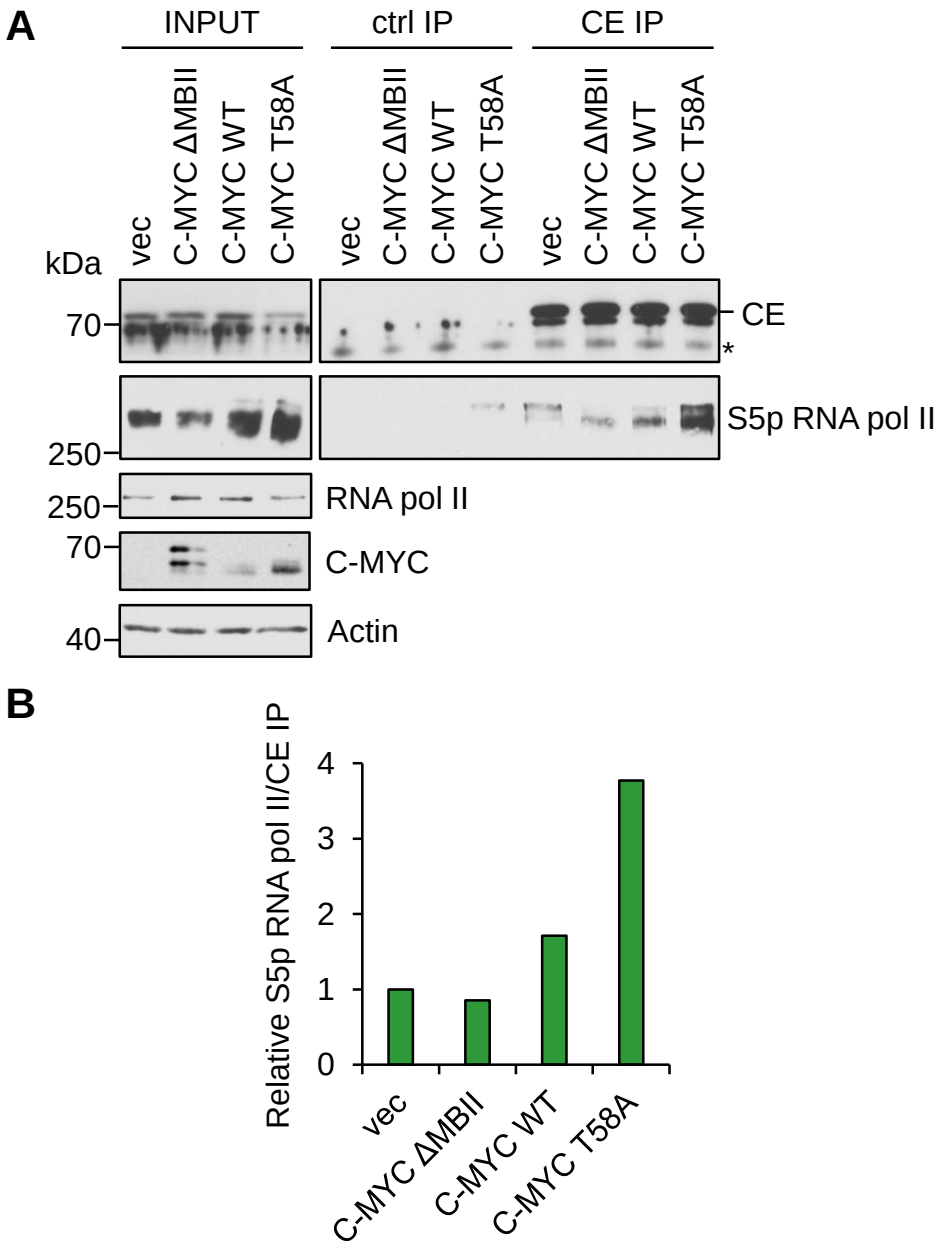

Figure 3.7: Probing C-MYC-dependent CE recruitment using C-MYC mutants. IMECs were stably transduced with empty vector (vec), *C-MYC WT, C-MYC ΔMBII* or *C-MYC T58A* constructs. CE IPs were performed for 2 hours from protein extracts. IPs using a GST tag antibody were performed as a control (ctrl IP). 19mg protein and 2µg antibody was used in each IP. 80% of eluate was loaded for S5p RNA pol II and CE Western blots (same gel). IP eluates were analysed on a 3-8% pre-cast gel. *non-specific/likely heavy chain. Individual experiment. (B) Densitometry was performed using ImageJ software to quantify the Western blot signal of S5p RNA pol II following CE immunoprecipitation, normalised to the CE IP Western blot signal. GST (control) IP signal was subtracted. All bands in the S5p RNA pol II WB are included in the quantification.

relationship between C-MYC activity and CE recruitment. To verify that this relationship is S5p RNA pol II-dependent, the effect of CDK7 inhibition on C-MYC-dependent CE recruitment should be analysed.

3.2.2 Investigating whether C-MYC regulates SPT5 modification

As previously mentioned, SPT5 possesses a CTR domain which is analogous to the RNA pol II CTD, and can be phosphorylated by TFIIH/CDK7 and P-TEFb/CDK9 (Figure 1.3) (Kim and Sharp, 2001; Larochelle et al., 2006). Since C-MYC increases the expression and recruitment of these kinases, it is possible that C-MYC could regulate SPT5 phosphorylation, which in turn may mediate its interaction with CE. In IMECs, C-MYC overexpression increased the abundance of a higher molecular weight form of SPT5 (Figure 3.8 A). Knocking down C-MYC by siRNA in HeLa cells diminished RNA pol II S5 and S2 phosphorylation, as expected, and decreased the presence of higher molecular weight SPT5 (Figure 3.8 B). This preliminary data indicates that C-MYC regulates SPT5 phosphorylation, although further studies are required to verify this result. Extracts could be treated with phosphatase to confirm that the higher molecular weight band is phosphorylated SPT5, and samples could be labelled with Phos-tag (Alpha Laboratories) to better resolve and analyse phosphorylated SPT5 on a gel.

3.2.3 C-MYC increases the CE guanylyltransferase activity associated with RNA pol II

Since binding of RNA pol II S5pCTD peptides allosterically stimulates CE guanylyltransferase activity in vitro and C-MYC increases S5 RNA pol II phosphorylation in cells, it was investigated whether C-MYC stimulated cellular

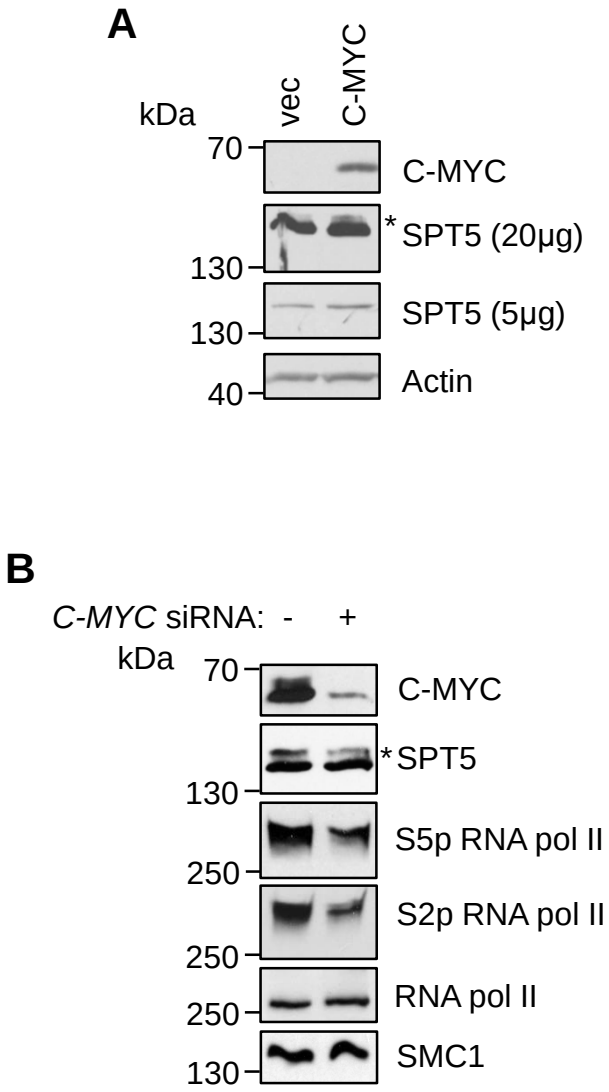

Figure 3.8: Investigating how C-MYC influences SPT5 modification. (A) IMEC/vec and IMEC/C-MYC protein extracts were analysed by SDS-PAGE and Western blotting. Indicated is the amount of protein loaded on the gel. Individual experiment. (B) HeLa cells were transfected with 50nM *C-MYC* siRNA or a non-targeting control (-). After 24 hours, protein was extracted and analysed as above. Individual experiment. *slower migrating SPT5.

CE guanylyltransferase activity. RNA 5' guanylylation is thought to be the rate-limiting step in basic guanosine cap synthesis since the specific activity of the CE triphosphatase is much greater than that for the guanylyltransferase (Shuman, 1995). GMP-binding assays were performed which have previously been used to analyse guanylyltransferase activity of recombinant CE in vitro (Ho and Shuman, 1999). Using GTP labelled on the alpha phosphate ([α-^{32}P]GTP) as a substrate for CE results in formation of a covalent CE-[^{32}P]GMP intermediate, which can be quantified and used as an approximation of CE guanylyltransferase activity (Figure 3.9 A). In previous studies, RNA pol II S5p-CTD stimulated the guanylyltransferase activity of CE by 1.5-, 2.8- or 4-fold when 2, 4 or 6 tandem S5pCTD repeats were used, respectively (relative to reactions with no CTD peptides) (Ho and Shuman, 1999). No stimulation was observed with non-phosphorylated CTD or S2pCTD repeats. Two tandem CTD repeats are necessary for the CE-RNA pol II interaction, which is likely because an additional contact is made between CE and Y1 of the following CTD heptad (Ghosh et al., 2011). Here, CE was immunoprecipitated from IMEC/vec and IMEC/C-MYC nuclear protein extracts. Nuclear fractionation was performed to exclude the activity of cytoplasmic re-capping by CE (Otsuka et al., 2009). After being incubated with [α-^{32}P]GTP for one minute, IPs were resolved by SDS-PAGE and [^{32}P]GMP incorporation quantified by phosphorimager. CE-GMP could be specifically immunoprecipitated and CE was the only detectable GMP-binding protein in CE IPs (Figure 3.9 B). Incubating reactions with [α-^{32}P]GTP for longer than 2.5 minutes decreased the CE-GMP signal, indicating that substrate turnover may be occurring (Figure 3.9 C). Therefore, IPs were treated with RNase prior to incubation with [α-^{32}P]GTP to prevent substrate turnover and preserve the CE-GMP intermediate in the reaction. Loading different

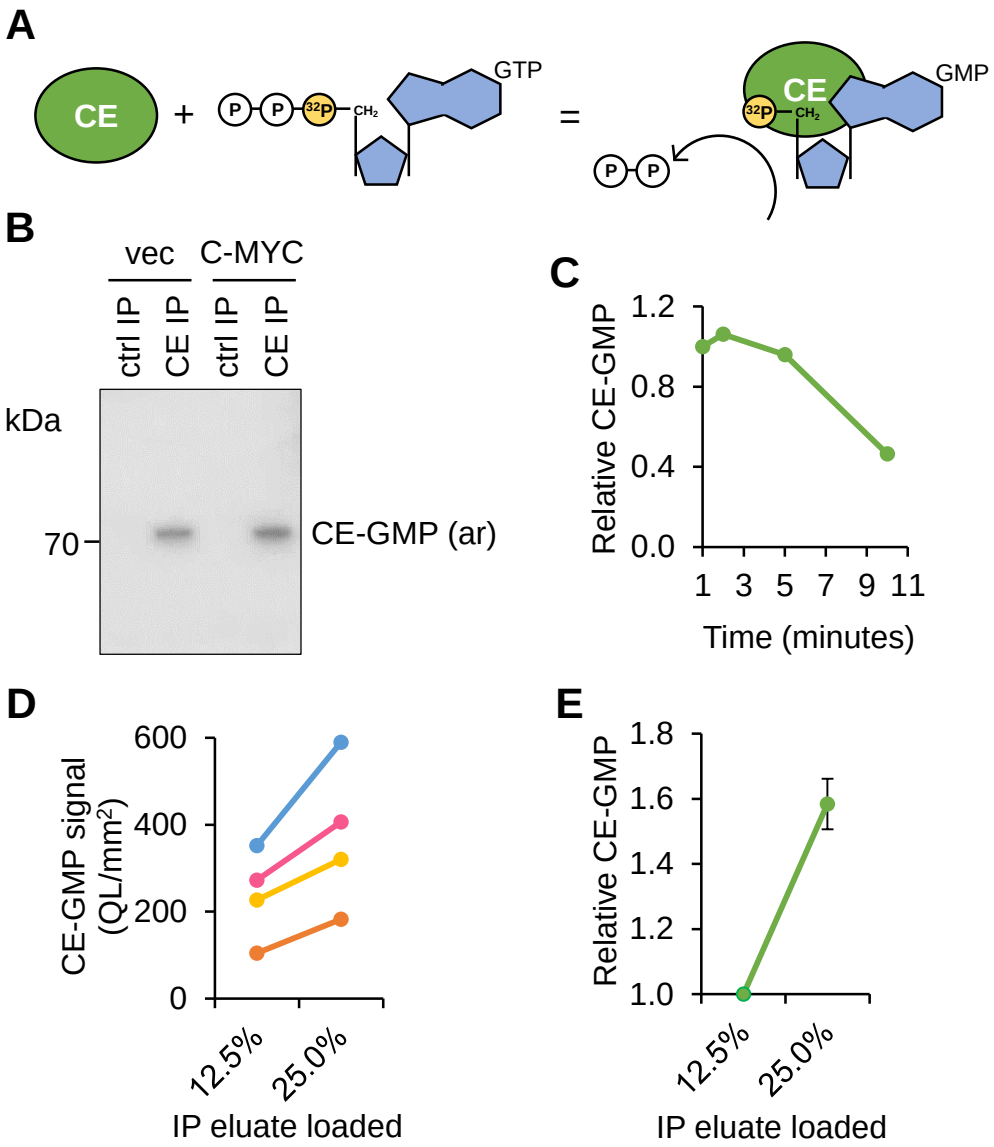

Figure 3.9: CE guanylyltransferase assays using cell extracts. (A) CE reacts with [α-^{32}P]GTP, yielding a ^{32}P-labelled CE-GMP intermediate which is used as an approximation of CE guanylyltransferase activity. (B) CE was immunoprecipitated from IMEC/vec and IMEC/C-MYC nuclear protein extracts. IPs using GST tag antibody were performed as a control (ctrl IP). Each IP contained 100µg protein and 0.5µg antibody. IPs immobilised on beads were incubated with [α-^{32}P]GTP reaction mix. Eluates were run on a gel which was then dried and analysed by phosphorimager for incorporation of ^{32}P. ar, autoradiograph. (C) CE IPs were incubated with [α-^{32}P]GTP reaction mix for the indicated times. Average values from two experiments (except 2.5 min time-point – individual experiment). (D) 12.5% or 25% of CE IP eluate from four independent samples were loaded to test the linearity of the assay. (E) As in (D) except expressed as average increase in signal. Error bars represent standard error of the mean, n=4.

amounts of CE IPs demonstrated that this method is semi-quantitative (Figure 3.9 D and E). C-MYC did not alter nuclear CE guanylyltransferase activity (Figure 3.10 A and B). However, only a fraction of CE co-purifies RNA pol II and SPT5 in IMECs (Figure 3.5), consistent with the majority of CE not being associated with RNA pol II chromatin complexes in the cell. CE catalyses co-transcriptional mRNA capping when engaged with transcription complexes, and therefore RNA pol II- and SPT5- bound protein fractions were analysed for guanylyltransferase activity. RNA pol II and SPT5 were immunoprecipitated from IMEC protein extracts and the co-purifying CE-GMP complexes were analysed (Figure 3.11). Consistent with CE co-immunoprecipitations, a relatively small amount of CE-GMP was co-purified with RNA pol II and SPT5 compared to with CE. There was more CE-GMP associated with RNA pol II than SPT5, illustrating that RNA pol II-bound CE is more abundant or catalytically active than that associated with SPT5. However, this does not exclude the possibility that the IP efficiencies were different, and as before the SPT5-bound CE-GMP could be indirectly interacting via RNA pol II. It was then investigated if C-MYC increased guanylyltransferase activity of RNA pol II-bound CE. RNA pol II was immunoprecipitated from IMEC/vec and IMEC/C-MYC protein extracts and the co-purified CE-GMP was the only GMP-binding protein detected (Figure 3.12 A). Interestingly, there was consistently less RNA pol II purified from IMEC/C-MYC extracts by immunoprecipitation compared to IMEC/vec under these experimental conditions. Since C-MYC does not typically cause a reduction in RNA pol II expression levels (Figures 3.4 B, 3.5 A and 3.6 A), RNA pol II may be more unstable when immunoprecipitated from IMEC/C-MYC extracts. This is likely a result of the long

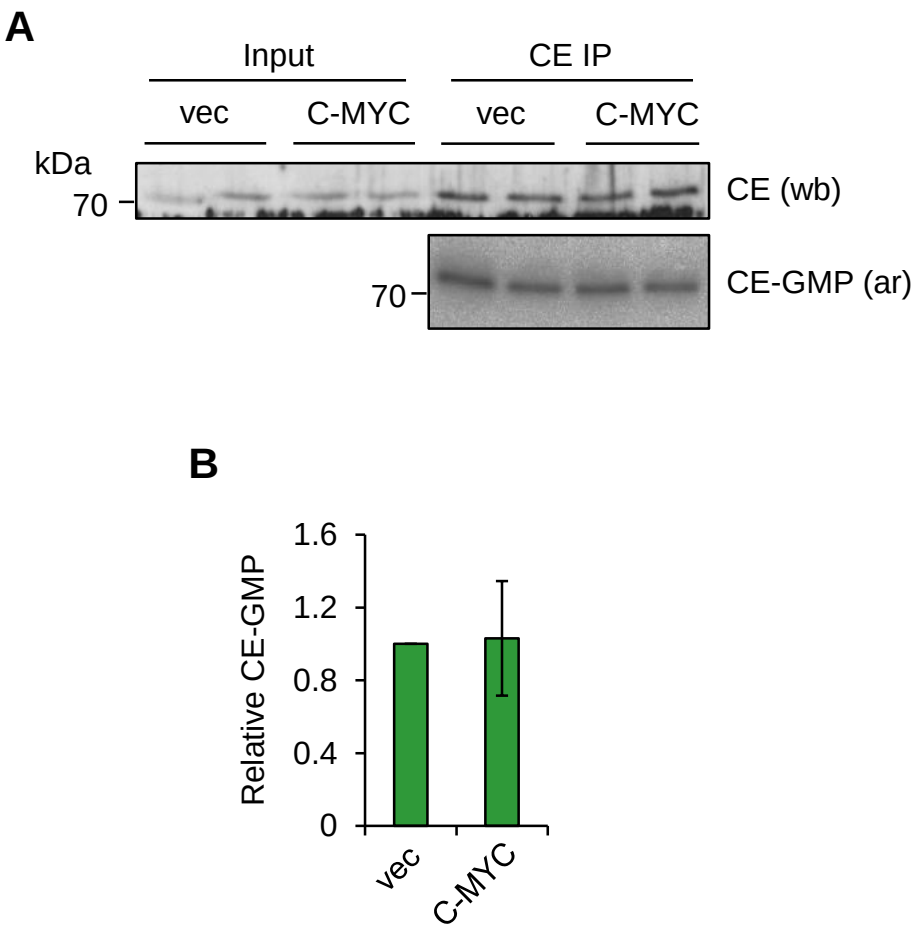

Figure 3.10: C-MYC does not alter total nuclear CE guanylyltransferase activity. (A) CE was immunoprecipitated from IMEC/vec and IMEC/C-MYC nuclear protein extracts for 2.5 hours. Technical duplicates were performed for each condition. Each IP contained 100µg protein and 0.5µg antibody. After being incubated with reaction mix, eluates were run on a gel which was then dried and analysed by phosphorimager for incorporation of ^{32}P. CE Western blots (wb) were performed as a loading control. ar, autoradiograph. (B) The signal obtained by phosphorimager was quantified using 2D densitometry in AIDA image analyser. Error bars represent standard error of the mean, n=5.

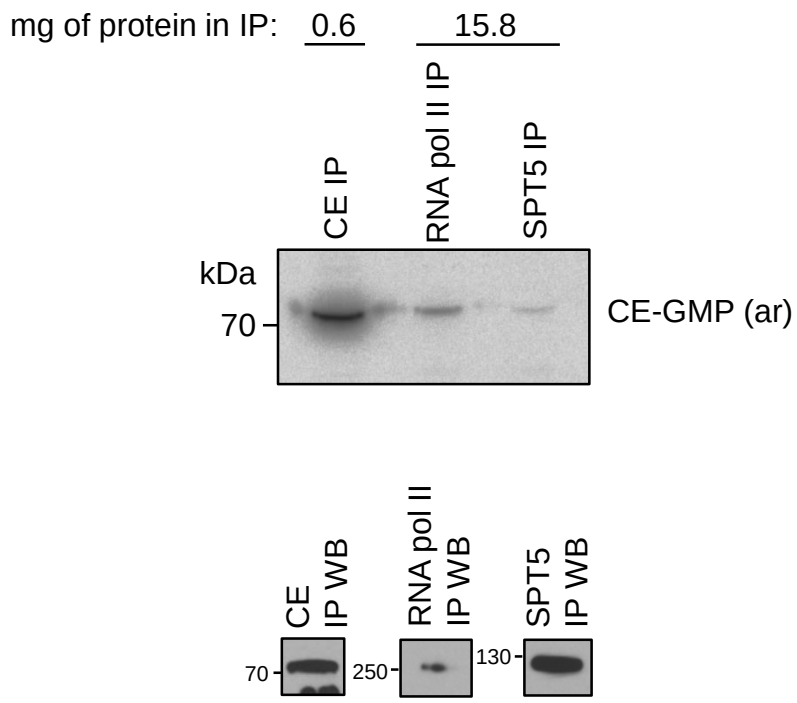

Figure 3.11: Comparison of total CE guanylyltransferase activity to that associated with RNA pol II and SPT5. CE, RNA pol II and SPT5 were immunoprecipitated from IMEC/vec protein extracts for 2.5 hours using 1µg, 2µg and 10µl of antibody, respectively. The CE IP contained 0.6mg of protein and the RNA pol II and SPT5 IPs contained 15.8mg. After IPs were incubated with reaction mix and eluted, 80% of eluates were run on a gel which was then dried and analysed by phosphorimager for incorporation of ^{32}P. 5% of eluates were run on a gel for analysis by Western blot to confirm that the proteins of interest were immunoprecipitated. ar, autoradiograph. Individual experiment.

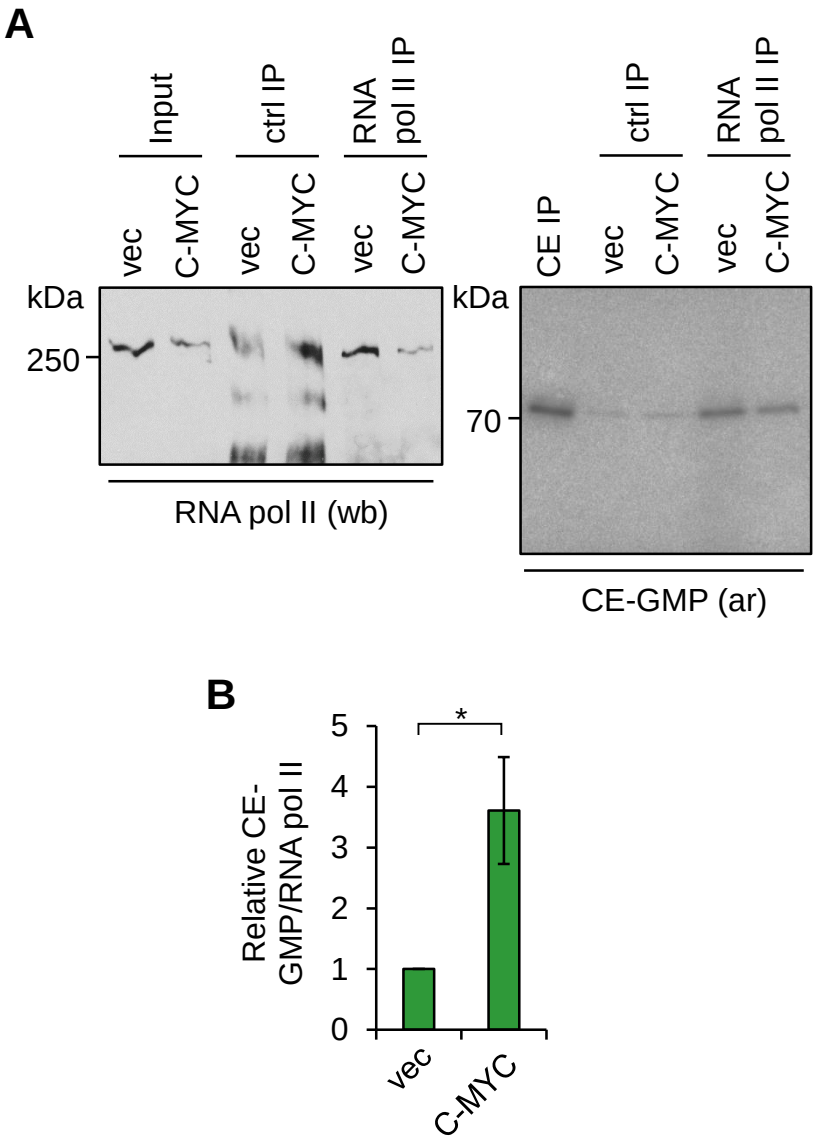

Figure 3.12: C-MYC overexpression increases RNA pol II-associated CE guanylyltransferase activity. (A) RNA pol II was immunoprecipitated from IMEC/vec and IMEC/C-MYC protein extracts overnight. IPs using HIF-1α antibody were performed as a control (ctrl IP). IPs contained 26.5mg protein and 4µg antibody. A CE IP was performed as a positive control with 100µg protein. IPs were incubated in reaction mix before being eluted. Samples were run on a gel which was then dried and analysed by phosphorimager for incorporation of ^{32}P (phos). RNA pol II Western blots (wb) were performed as a loading control. ar, autoradiograph. (B) The signal obtained by phosphorimager was quantified using 2D densitometry in AIDA image analyser. The CE-GMP signal was normalised to the RNA pol II Western blot signal, quantified by densitometry using ImageJ. Error bars represent standard error of the mean, n=4. Significance was calculated by Student's t-test; *p≤0.05.

immunoprecipitation time, as shorter RNA pol II immunoprecipitations from IMEC/vec and IMEC/C-MYC protein extracts purify equal amounts of RNA pol II (Figure 3.6 A), but these shorter immunoprecipitations were not sufficient to detect CE-GMP (data not shown). To interpret the CE-GMP signal relative to RNA pol II, the CE-GMP signal was normalised to the RNA pol II IP Western blot signal (Figure 3.12 B). C-MYC increased relative RNA pol II-bound CE guanylyltransferase activity. Although this increase is not additive to the increased interaction of CE with RNA pol II (Figure 3.5 B), both this assay and IP-Western blots are only semi-quantitative which makes it difficult to compare the CE-RNA pol II interaction with RNA pol II-associated CE activity. Nonetheless, C-MYC increases RNA pol II-associated CE guanylyltransferase activity, whether that is solely due to increased CE recruitment or also allosteric stimulation of CE.

3.2.4 C-MYC regulates CE recruitment to C-MYC target genes

Since C-MYC increased the interaction of CE with RNA pol II and SPT5, it was investigated whether *C-MYC* overexpression in IMECs increased the recruitment of CE to C-MYC target genes. Although CE does not directly interact with DNA, RNA pol II and SPT5 do and thus it has the potential to be crosslinked to chromatin. Therefore, CE chromatin immunoprecipitation (ChIP) was performed in IMECs, but no binding above background levels could be detected (data not shown). As an alternative, HeLa cells were used which are routinely used for ChIP assays and express endogenously deregulated C-MYC. *C-MYC* siRNA was optimised, and an associated decrease in S5p RNA pol II levels was observed after 24 hours at higher siRNA concentrations (Figure 3.13 A and B, 24 hour knockdown using 50nM *C-MYC* siRNA selected). A selection

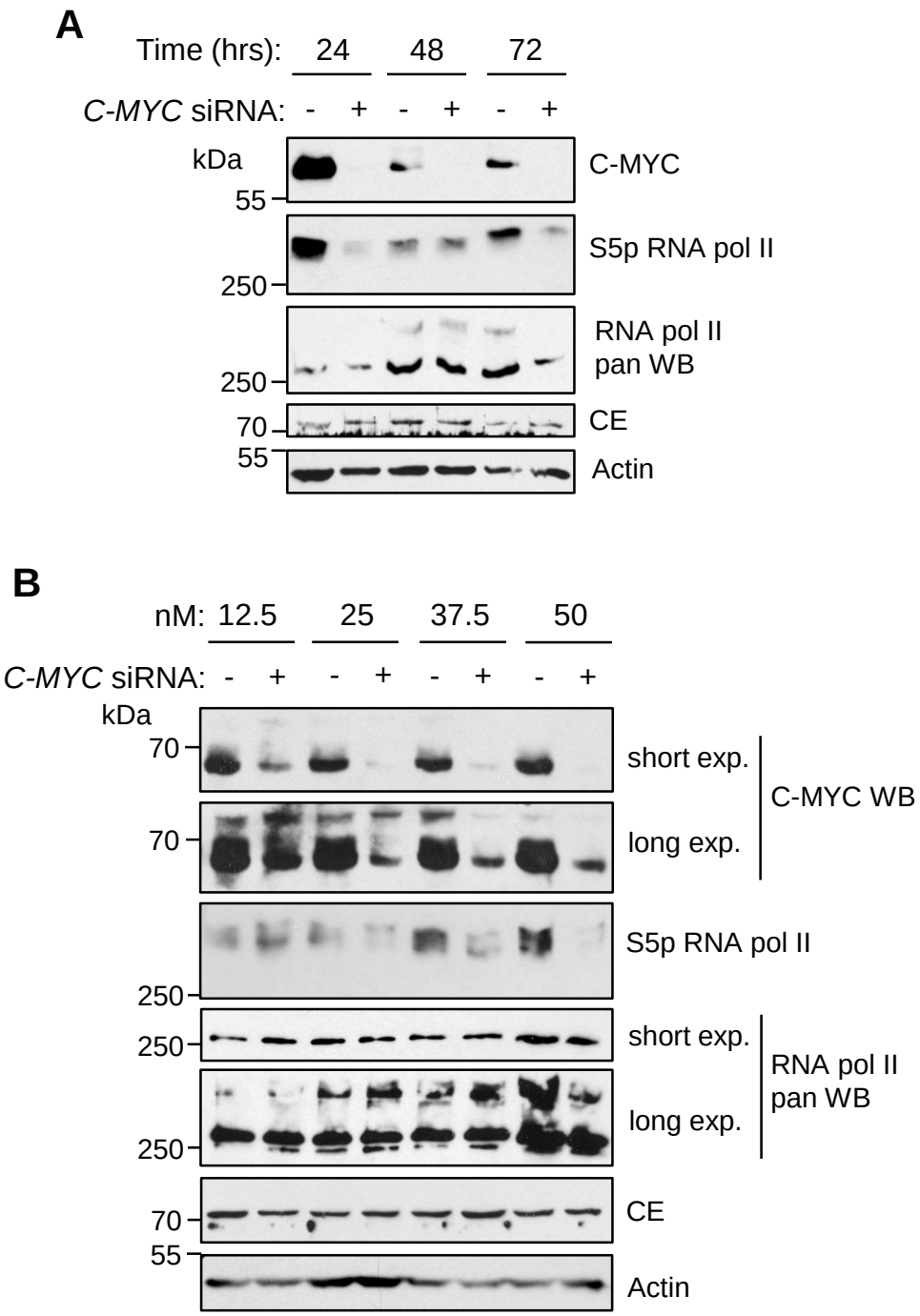

Figure 3.13: Optimisation of siRNA-mediated C-MYC depletion in HeLa cells. (A) HeLa cells were transfected with 50nM C-MYC siRNA or a non-targeting control (-). 24, 48 or 72 hours later, protein was extracted and analysed by SDS-PAGE and Western blotting. Individual experiment. (B) HeLa cells were transfected with 12.5-50nM C-MYC siRNA or a non-targeting control (-) as indicated. After 24 hours, protein was extracted and analysed as above. Individual experiment.

of C-MYC target genes was analysed by RT-qPCR to identify those which were most responsive to C-MYC knockdown (Figure 3.14 A and B). Only *NCL* and *NME1* expression was substantially depleted after 24 hours (Figure 3.14 A), but most of the target genes were diminished after 48-72 hours (Figure 3.14 B), which probably reflects the stability of the C-MYC target gene mRNAs. *NCL*, *FBL* and *NME1* transcripts were the most significantly reduced in response to C-MYC suppression and thus the recruitment of CE to these genes was analysed by ChIP. Sonication of samples was optimised to yield chromatin fragment sizes suitable for ChIP (200-500 bp - 30 minutes sonication selected) (Figure 3.15 A) CE binding to regions surrounding the TSS and C-MYC-binding sites was analysed by qPCR (Figure 3.15 B and C). As previously observed, it is not uncommon for C-MYC binding sites to occur within the first intron (Dang, 2012). The binding of CE to the TSS of the constitutively expressed gene *GAPDH* was also analysed. A low, yet above background, signal could be detected for CE binding to the analysed regions in control treated cells (<0.2% of input DNA recovered bound to CE, data not shown). CE was most abundant at the TSS of C-MYC target genes or proximally upstream, and there was a reproducible trend towards less CE recruitment upon C-MYC depletion, although this did not achieve statistical significance. C-MYC knockdown resulted in a significant decrease in CE binding to *NCL* and *FBL* genes downstream of their transcription start sites. Additionally, the recruitment of CE to the TSS of *NME1* and *GAPDH* was reproducibly diminished by C-MYC knockdown, but this again is not statistically significant. Although *GAPDH* transcript levels were not affected by C-MYC knockdown in HeLa cells (data not shown), the *GAPDH* gene contains a non-canonical E-box (CGCGTG) ~300 bases upstream of the TSS, and a canonical E-box (CACGTG) ~800 bases

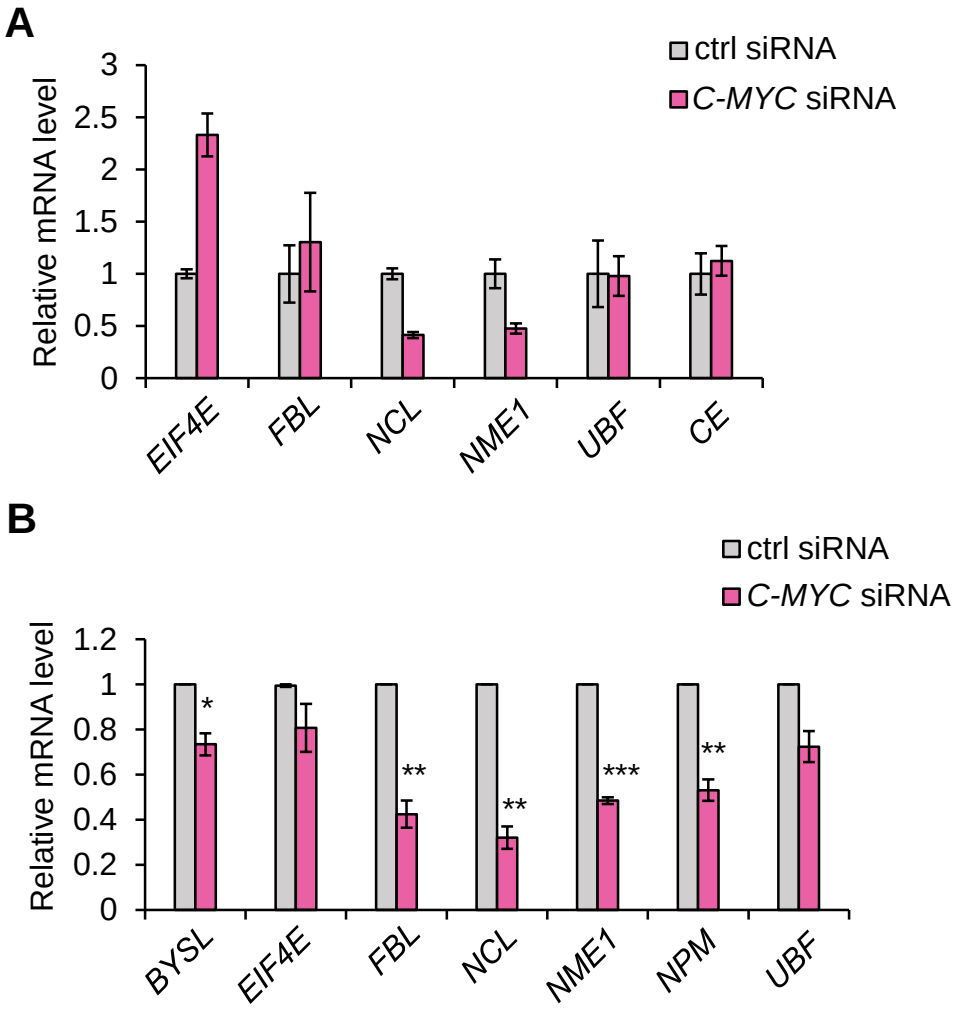

Figure 3.14: Verification of C-MYC target genes in HeLa cells. (A) HeLa cells were transfected with 50nM *C-MYC* siRNA or a non-targeting control (ctrl). After 24 hours, RNA was extracted and analysed by qRT-PCR. Transcript expression is normalised to that of GAPDH. Error bars represent standard error of the mean, n=3 (technical replicates). (B) As in (A) but analysed after 48-72 hours. Error bars represent standard error of the mean, n≥3. Significance was calculate by Student's t-test, ***p≤0.001; **p≤0.01; *p≤0.05.

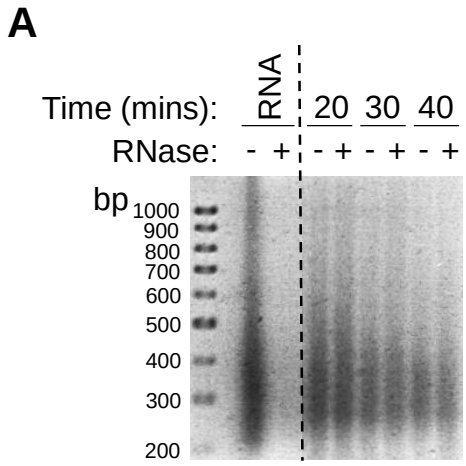

Figure 3.15: C-MYC depletion diminishes CE recruitment to C-MYC target genes. (A) HeLa cells were cross-linked for 15 minutes before being lysed and treated with and without RNase prior to sonication to ensure the nucleic acid visualised was DNA. RNA was used as a positive control for RNase treatment. Lysates were then subject to 30 seconds on/off cycles of sonication for the indicated times. Samples are from the same gel and same exposure, indicated by the dashed line. Individual experiment. Figure continued overleaf.

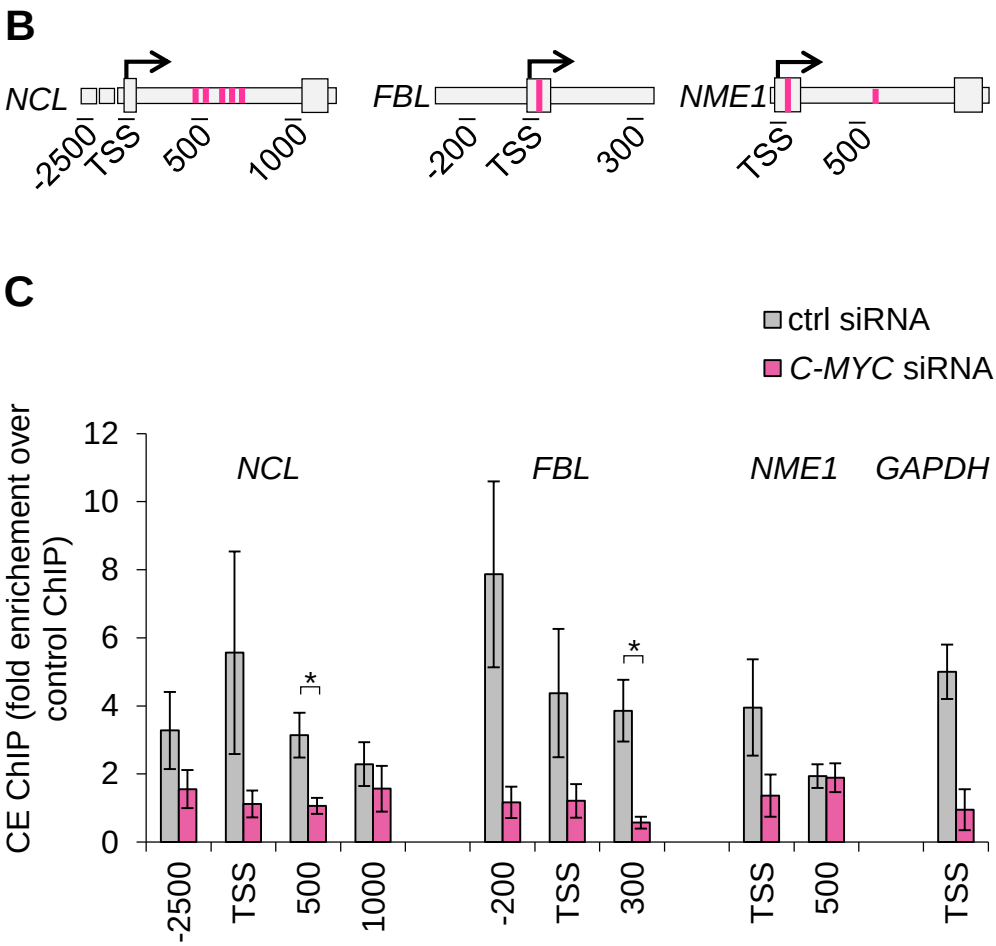

Figure 3.15 continued: C-MYC depletion diminishes CE recruitment to C-MYC target genes. (B) Schematics depicting regions of primer amplification used for analysis for CE ChIP DNA, relative to the TSS (arrow) and E-box sequence(s) (pink band). (C) HeLa cells were transfected with 50nM *C-MYC* siRNA or non-targeting control (ctrl) and were cross-linked after 24 hours. Lysates were sonicated to shear chromatin before performing CE immunoprecipitation. IPs with no antibody were included as a control. DNA was purified from eluates and then analysed by qPCR for C-MYC target gene or *GAPDH* DNA. CE ChIP signal is expressed as fold enrichment over no antibody ChIP signal. Error bars represent standard error of the mean, n≥3. Significance was calculated by Student's t-test; *p≤0.05.

downstream of the TSS (Figure 1.6), which might explain why CE recruitment to *GAPDH* is C-MYC-responsive. CE binding to the furthermost downstream regions analysed of *NCL* and *NME1* was low (only ~2-fold over background) and was not C-MYC-responsive. Of note, although in most studies C-MYC is reported to increase RNA pol II elongation and not RNA pol II recruitment, the latter has been reported (Walz et al., 2014). Therefore, it would be favourable to analyse RNA pol II occupancy on these genes upon C-MYC knockdown to determine if this contributes to reduced CE recruitment. In summary, C-MYC promotes the recruitment of CE to classical C-MYC target genes and probably other genes.

3.3 Discussion

3.3.1 Summary

In this chapter, it was demonstrated that C-MYC deregulation increased the recruitment of CE to RNA pol II and SPT5. This was associated with stimulation of RNA pol II-bound CE guanylyltransferase activity. Inhibiting C-MYC expression abolished CE recruitment to C-MYC target genes proximal to the TSS. In summary, C-MYC regulates the recruitment of CE to transcription complexes near the 5' end of genes, which could be used to drive or enhance C-MYC target gene expression.

3.3.2 Potentially distinct mechanisms for CE and RNMT recruitment

RNA pol II CTD phosphorylation is thought to mediate the timely recruitment of various factors during transcription. RNA pol II S5 phosphorylation occurs after RNA pol II has associated with transcription start sites during initiation. S5p RNA pol II recruits CE to nascent pre-mRNA which are capped as they emerge from RNA pol II at 18-25 nucleotides in length (Coppola et al., 1983; Martinez-Rucobo et al., 2015; Moteki and Price, 2002). In vitro and in cells, an interaction between RNMT and RNA pol II CTD cannot be detected (Figure 3.1; Aregger and Cowling, 2013; Pineda et al., 2015; Shatkin and Manley, 2000). Logically, the recruitment of RNMT via a different mechanism could allow for it be recruited after CE in transcription, since basic guanosine cap addition is a prerequisite for cap methylation. Indeed, in vitro, RNMT methylates nascent pre-mRNAs which are at least 35 nucleotides long, and the time taken for cap methylation to occur is six-fold longer than for guanosine cap addition (Moteki and Price, 2002). ChIP in a previous study

showed that the spatial occupancy of CE and RNMT across several genes was much the same, although temporal information concerning recruitment may be lost since binding correlates with RNA pol II pausing (Glover-Cutter et al., 2008). Alternatively, perhaps both are recruited within a similar timeframe to ensure seamless catalysis of the capping reactions, but via different proteins to prevent competition for binding.

3.3.3 The CE and RNA pol II interaction

Results presented here showed that a very small proportion of CE purified with RNA pol II and transcription complexes from cells. This is observed by CE-RNA pol II co-IPs, RNA pol II-bound CE guanylyltransferase assays and CE ChIPs. There are several possible interpretations of this observation. It could reflect a transient interaction. In favour of this, CE is thought to dissociate from RNA pol II once capping occurs (Martinez-Rucobo et al., 2015). On the other hand, it might indicate that there is a great excess of CE relative to RNA pol II in the cell, such that CE abundance is not limiting. This could potentially allow for rapid tuning of CE recruitment via modulation of RNA pol II CTD S5 phosphorylation.

3.3.4 C-MYC increases CE recruitment

The finding that C-MYC regulates CE recruitment is an exciting observation which demonstrates that C-MYC regulates mRNA capping at multiple levels. As previously discussed, C-MYC-mediated RNA pol II phosphorylation upregulates RNMT recruitment (Aregger and Cowling, 2013; Cowling and Cole, 2007b; Posternak et al., 2017). Moreover, the C-MYC-target gene SAHH catalyses removal of the inhibitory by-product of RNMT activity

(SAH) (Fernandez-Sanchez et al., 2009). However, whether C-MYC regulated the prior steps in cap catalysis was not clear. Observations in this chapter show that C-MYC co-ordinates both CE and RNMT to drive effective mRNA capping. C-MYC increasing 5' mRNA guanylylation – which is critical for transcript stability – via CE recruitment is consistent with C-MYC activation globally enhancing mRNA stability in other studies (Dang et al., 2017; Hsu et al., 2015). Since C-MYC deregulation causes broad changes in cellular metabolism, including increased nucleotide biosynthesis (Barfeld et al., 2015; Liu et al., 2008), it may be that C-MYC also regulates CE via additional routes such as modulating GTP availability or upregulating removal of the inhibitory by-product of RNA guanylylation (PPi). Notably, C-MYC is likely not the only transcription factor that upregulates mRNA capping, but since C-MYC globally enhances transcription it may be particularly reliant on CE recruitment to enhance target gene expression.

3.3.5 C-MYC increases CE activity

Although C-MYC increased CE guanylylation associated with cellular RNA pol II, it is not clear if this is solely indicative of more CE recruitment or if C-MYC-dependent RNA pol II phosphorylation also allosterically activates CE. By removing RNA in IPs prior to the CE guanylylation assay it was hoped this would prevent substrate turnover, thereby preserving the CE-GMP interaction, but it is possible that substrate RNA bound to RNA pol II and CE was masked from RNase degradation. Furthermore, CE guanylylation is a reversible reaction, and the kinetics of the forward and back reactions in this experiment are not clear. Perhaps using more limiting concentrations of GTP would have been favourable to analyse CE-GMP formation. Moreover, since the transfer of

GMP to RNA is slower than GTP hydrolysis by CE (Shuman, 1995), it may be a more limiting step in RNA 5' guanylylation. It would therefore be interesting to analyse how C-MYC deregulation affects the ratio of guanosine-capped to uncapped mRNA in the cell. However, this could prove difficult to analyse in cells since uncapped mRNAs are unstable.

3.3.6 Specificity of C-MYC dependent CE recruitment

As determined by co-IPs, the C-MYC-mediated increase in CE recruitment occurs on a global scale. This is consistent with C-MYC globally increasing RNA pol II phosphorylation, globally enhancing mRNA stability, and the observation that C-MYC binds to the promoters of all actively expressed genes in certain systems (Cowling and Cole, 2007b; Hsu et al., 2015; Lin et al., 2012b; Nie et al., 2012; Sabo et al., 2014). It would be of interest to conduct ChIP-sequencing (ChIP-seq) analysis of CE, C-MYC and RNA pol II to determine if CE is preferentially recruited to C-MYC target genes and/or highly transcribed genes. Furthermore, it would be interesting to modulate other transcription factors which activate genes via similar mechanisms to C-MYC (i.e. influence RNA pol II pause release rather than recruitment) such as NF-κB and HIF1α to determine whether they also regulate CE recruitment to their target genes (Liu et al., 2015). Analysis of a select number of genes in this study indicated that CE recruitment is influenced by C-MYC. In yeast, TFIIH (containing a CDK7 homologue) is recruited to subsets of genes which mediates transcript-specific capping, and there is some evidence that this is governed by specific transcription factors (Viladevall et al., 2009). This indicates that CE may exhibit a degree of specificity. However, it should be noted that changes in CE recruitment may not necessarily reflect changes in gene

expression, as can be observed for changes in C-MYC recruitment for example (Kress et al., 2015). To this end, in the following chapter it will be investigated if CE regulates the expression of C-MYC target genes.

Chapter 4 : mRNA capping enzyme regulates C-MYC and C-MYC target genes

4.1 Introduction

C-MYC induces expression of its protein-coding target genes by binding promoter regions and enhancing RNA polymerase II transcription. C-MYC mediates these changes through several mechanisms, including increasing the recruitment of histone acetyltransferases (HATs), which promote histone acetylation and thus create a permissive chromatin environment (Frank et al., 2003; McMahon et al., 2000; Thomas et al., 2015a). In addition, C-MYC increases recruitment of RNA pol II CTD kinases, increasing S5 and S2 phosphorylation thereby enhancing the transition of paused RNA pol II molecules into processive elongation (Cowling and Cole, 2007b; Lin et al., 2012b; Rahl et al., 2010). RNA pol II pausing is controlled by DSIF and NELF, and release of RNA pol II from the pause site is a rate-limiting step in transcription (Adelman and Lis, 2012; Henriques et al., 2013; Liu et al., 2015; Min et al., 2011; Smith and Shilatifard, 2013). It is thought that pausing might function as temporal window for the recruitment of mRNA capping machinery and/or a checkpoint to ensure nascent RNA molecules are properly capped before transcription resumes. In addition, CE recruitment has a putative role in alleviating the RNA pol II pause (Mandal et al., 2004). Taken together with the role of C-MYC in RNA pol II pausing and with findings in the previous chapter, it is possible that C-MYC drives recruitment of CE to its target genes in order to optimise their expression. CE recruitment has the potential to both promote

mRNA capping of C-MYC target gene transcripts and enhance transcriptional pause release.

In this chapter, it was investigated whether CE influenced the expression of C-MYC target genes in the presence of basal or overexpressed C-MYC levels.

4.2 Results

4.2.1 CE is required for C-MYC expression

In order to study how CE affected C-MYC target genes, CE was depleted using siRNA. The siRNA oligo, siRNA concentration and length of knockdown was optimised in IMEC/vec and IMEC/C-MYC; a higher concentration of CE siRNA 2 was used since it does not work as well as CE siRNA 1 (Cowling lab, personal communication) (Figure 4.1 A-C). Using the selected conditions (72 hour knockdown using 50nM CE siRNA 1), CE was equivalently depleted in both cell lines at the mRNA and protein level (Figures 4.1 C and 4.2 A). Unexpectedly, CE knockdown resulted in a substantial (~40%) reduction in C-MYC protein levels, which occurred to an equal extent with endogenous C-MYC in IMEC/vec and exogenous C-MYC in IMEC/C-MYC (Figure 4.2 A-C). Note that the variation in this trend (Figure 4.2 B) lies mostly in the fluctuation of ectopic C-MYC levels, since the change in C-MYC expression upon CE knockdown is consistent (Figure 4.2 C). To confirm that this was a specific effect of CE knockdown, a second round of retroviral infection was used to stably express constructs encoding siRNA-resistant CE-GFP (CE-GFP WBL), regular CE-GFP or GFP alone. The CE-GFP WBL construct contains wobble codons/silent mutations, such that the mRNA sequence is not complementary to the siRNA but the protein sequence is the same as the wild-type. *CE-GFP WBL* mRNA was not significantly affected by *CE* siRNA (Figure 4.3 A). CE-GFP WBL protein was not affected by CE depletion, whereas endogenous CE and regular CE-GFP were depleted (Figure 4.3 B). CE-GFP WBL expression rescued the C-MYC expression defect in response to CE knockdown in

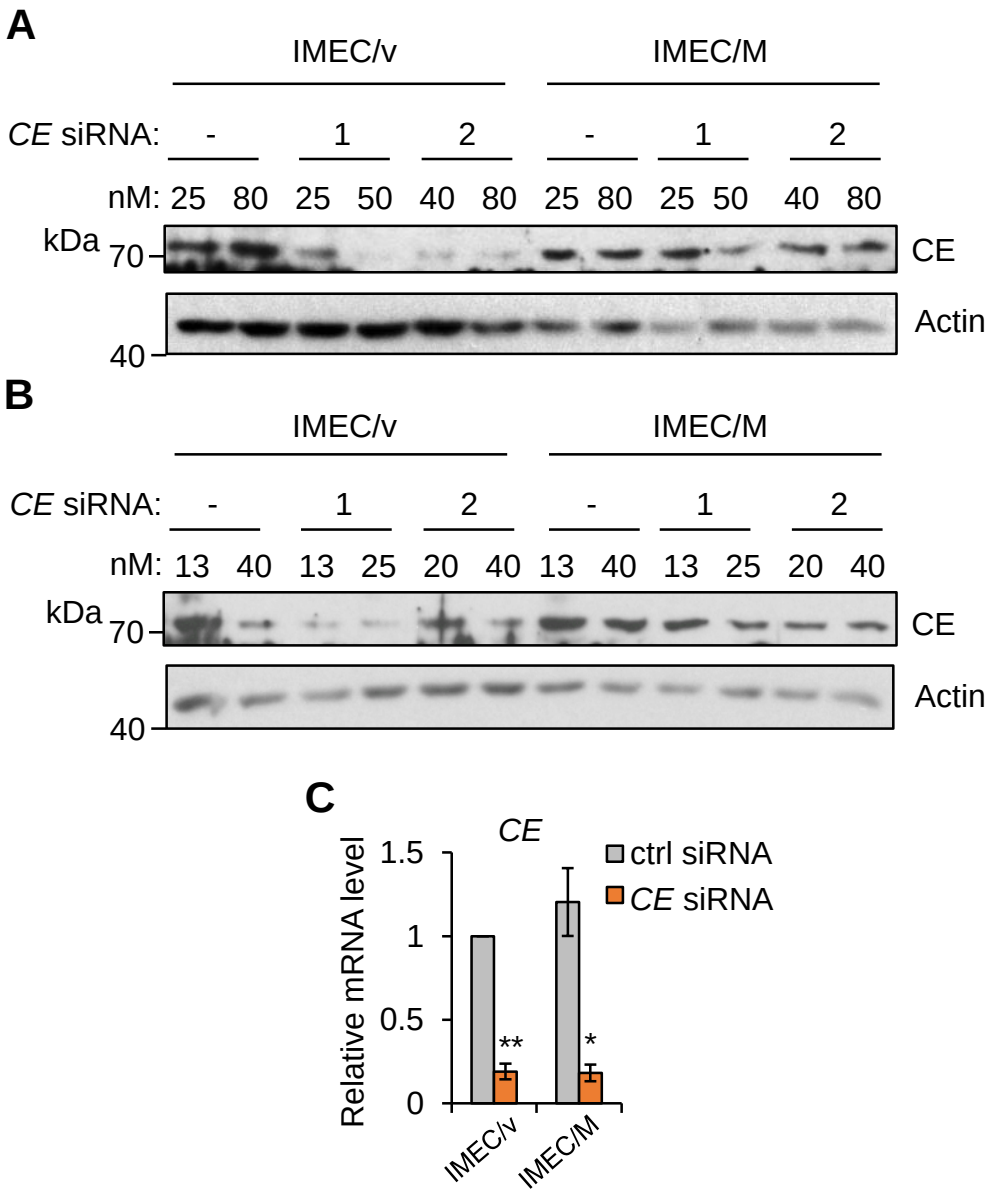

Figure 4.1: Optimisation of *CE* siRNA-mediated knockdown in IMECs. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with 25-80nM of non-targeting control (-), *CE* siRNA 1 or *CE* siRNA 2 as indicated. Higher concentrations of *CE* siRNA 2 were used following personal communication that it is not as efficient as *CE* siRNA 1 (Cowling lab). 48 hours later, protein was extracted and analysed by SDS-PAGE and Western blotting. Individual experiment. (B) As in (A) but cells transfected with 12.5-40nM siRNA and analysed after 72 hours. (C) IMEC/vec and IMEC/C-MYC were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (ctrl). After 72 hours, RNA was extracted and analysed by qRT-PCR. Transcript level is normalised to that of *GAPDH*. Error bars represent standard error of the mean, n=3. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01.

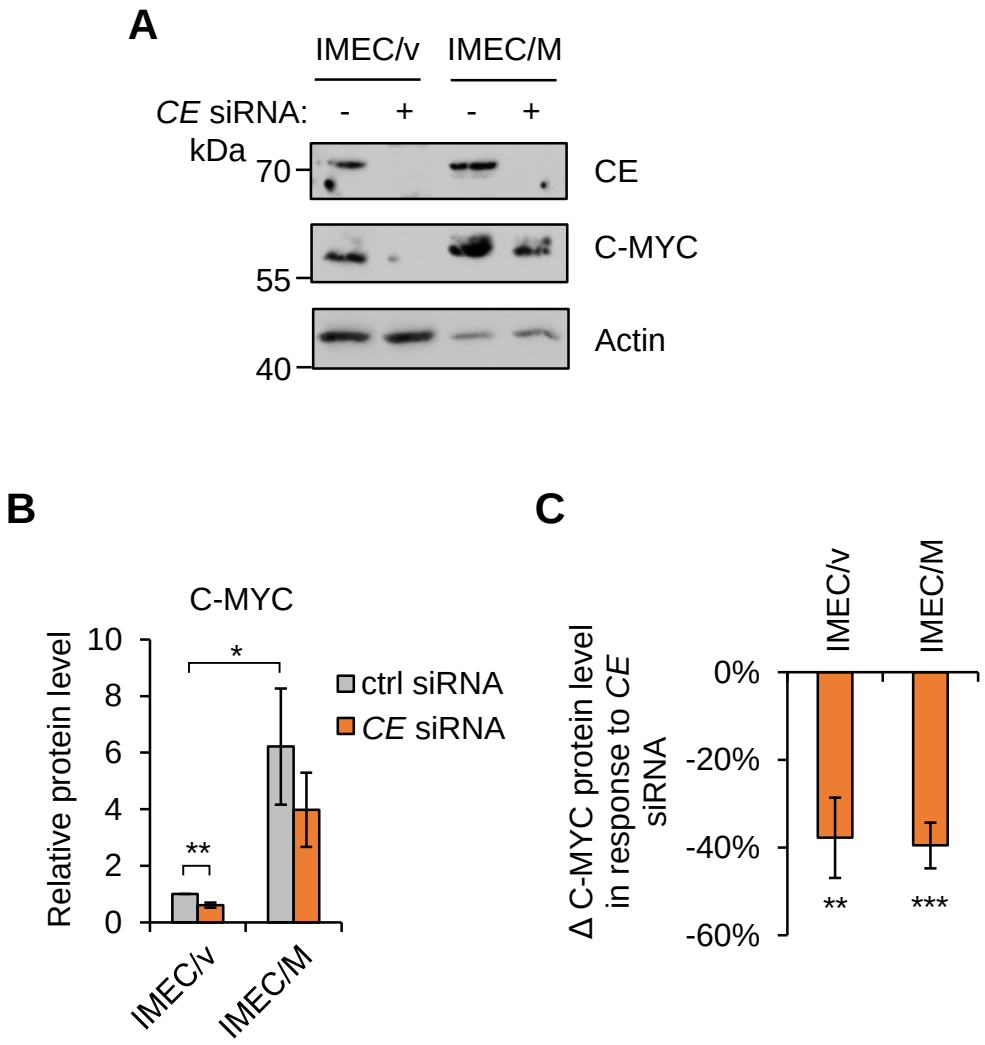

Figure 4.2: CE is required for C-MYC protein expression. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting. (B) Densitometry was performed using ImageJ software to quantify the C-MYC Western blot signal (normalised to actin) following transfection of *CE* siRNA 1 or non-targeting control (ctrl) as above. Error bars represent standard error of the mean, n≥6. (C) As in (B) but expressed as change (Δ) in expression following CE depletion relative to that in non-targeting control. Error bars represent standard error of the mean, n≥6. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01, ***≤0.001.

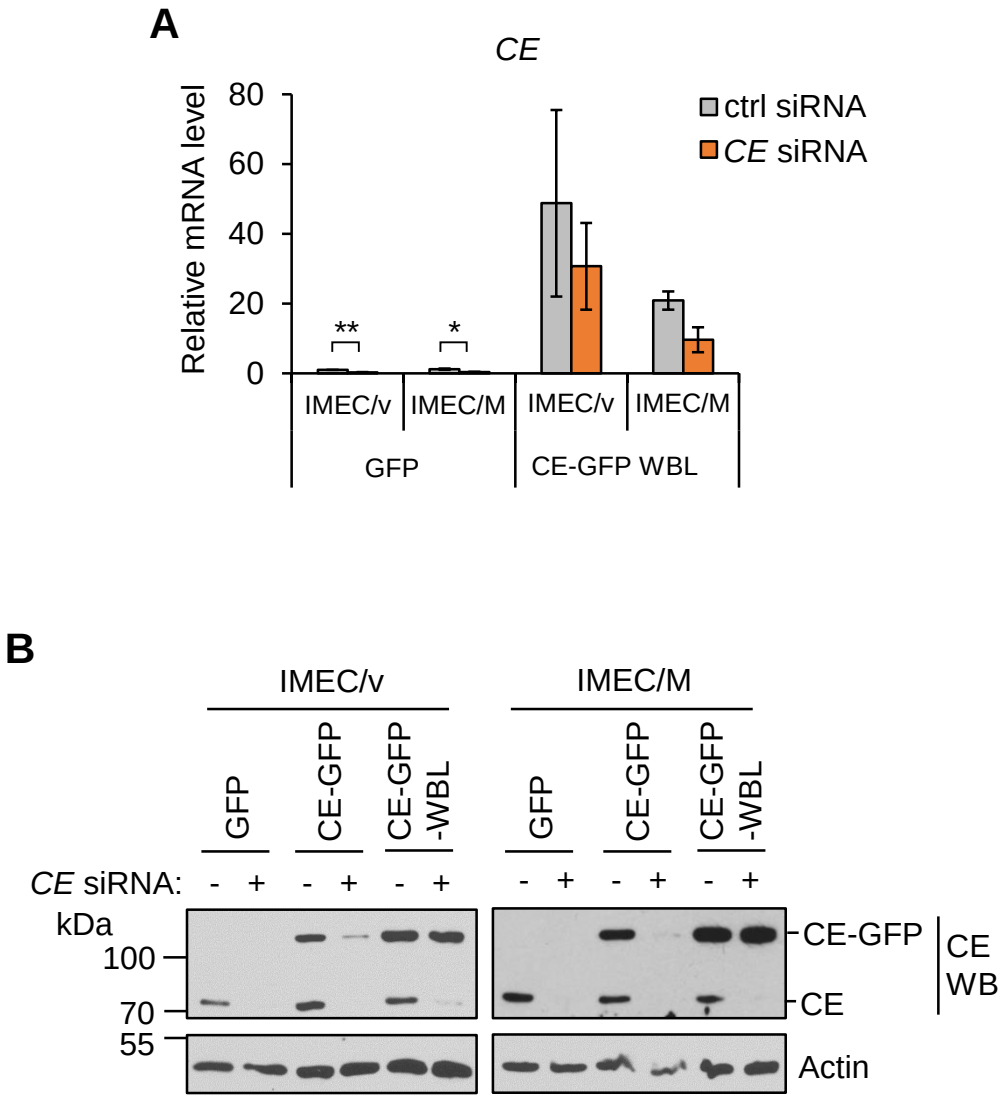

Figure 4.3: Generation of siRNA-resistant CE-GFP cell lines. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) stably transduced with GFP or siRNA-resistant CE-GFP (CE-GFP WBL) were transfected with 50nM CE siRNA 1 or non-targeting control siRNA (-). RNA was extracted from cells after 72 hours and analysed by RT-qPCR. Transcript level is normalised to that of *GAPDH*. Error bars represent standard error of the mean, n=3. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01. (B) IMEC/vec and IMEC/C-MYC stably transduced with GFP, CE-GFP or CE-GFP WBL were transfected with 50nM CE siRNA 1 or non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting.

IMEC/C-MYC (Figure 4.4), indicating that the effect of *CE* siRNA on C-MYC expression is specific.

Since mRNA capping can regulate several steps in gene expression, *C-MYC* mRNA levels were investigated to gain insight into how CE regulates C-MYC (Figure 4.5). Consistent with C-MYC undergoing autorepression above a certain expression threshold (Cleveland et al., 1988; Penn et al., 1990), endogenous *C-MYC* mRNA is repressed in IMEC/C-MYC. Both endogenous *C-MYC* in IMEC/vec and exogenous *FLAG-C-MYC* in IMEC/C-MYC were depleted in response to *CE* siRNA. However, despite endogenous and exogenous C-MYC protein levels being equivalently depleted, exogenous *C-MYC* mRNA is more sensitive to CE depletion. *C-MYC* mRNA in HeLa cells – which is highly expressed (Adey et al., 2013) – was also substantially reduced by *CE* siRNA. This is indicative of a transcriptional, stability or processing defect of *C-MYC* mRNA. Exogenous *C-MYC* is sensitive to CE depletion despite it lacking its endogenous promoter and introns, suggesting a decrease in mRNA stability is most likely.

Since C-MYC regulates RNA pol II phosphorylation, it was investigated whether the reduction in C-MYC expression as a result of CE depletion caused reduced S5 and S2 RNA pol II phosphorylation in IMECs (Figure 4.6 A and B). C-MYC increased S5 and S2 phosphorylation (Figure 4.6 B), as in previous studies. CE depletion caused fluctuations in both S5 and S2 phosphorylation, but no consistent trends were observed. This could be due to conflicting mechanisms, for example RNA pol II phosphorylation may be downregulated as a result of lower C-MYC levels, but upregulated via a distinct mechanism to compensate for lack of CE recruitment. To help determine the direct effect of

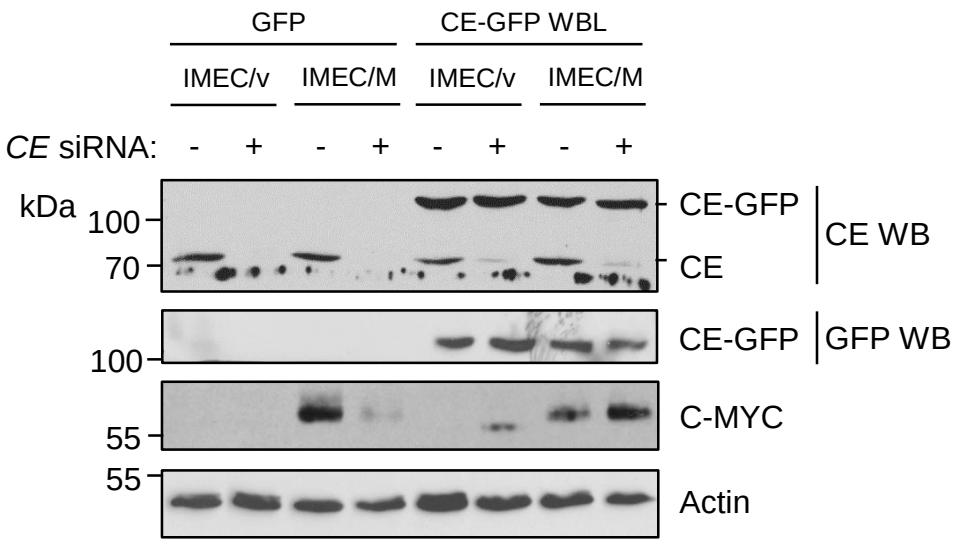

Figure 4.4: siRNA-resistant CE-GFP rescues the C-MYC expression defect upon CE knockdown in IMECs. IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) stably expressing siRNA-resistant CE-GFP (CE-GFP WBL) or GFP alone were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting. Representative of two independent experiments.

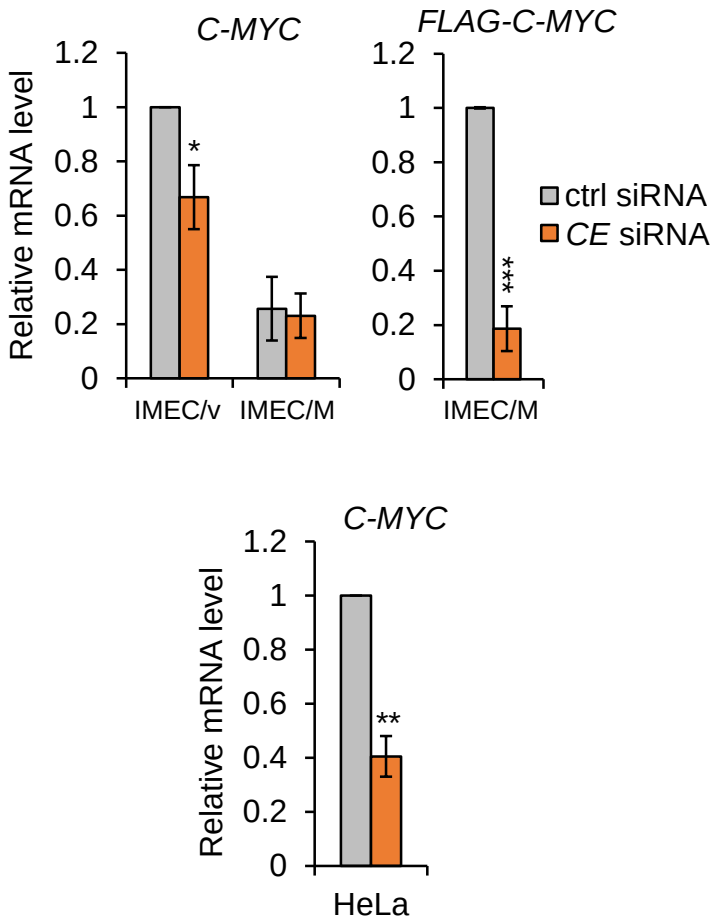

Figure 4.5: C-MYC mRNA levels are reduced upon CE knockdown. IMEC/vec (IMEC/v), IMEC/C-MYC (IMEC/M) and HeLa cells were transfected with 50nM *CE* siRNA 1 or non-targeting control (ctrl) siRNA. RNA was extracted from cells after 72 hours and analysed by RT-qPCR. Transcript level is normalised to that of *GAPDH*. Error bars represent standard error of the mean, n≥4. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01; ***p≤0.001.

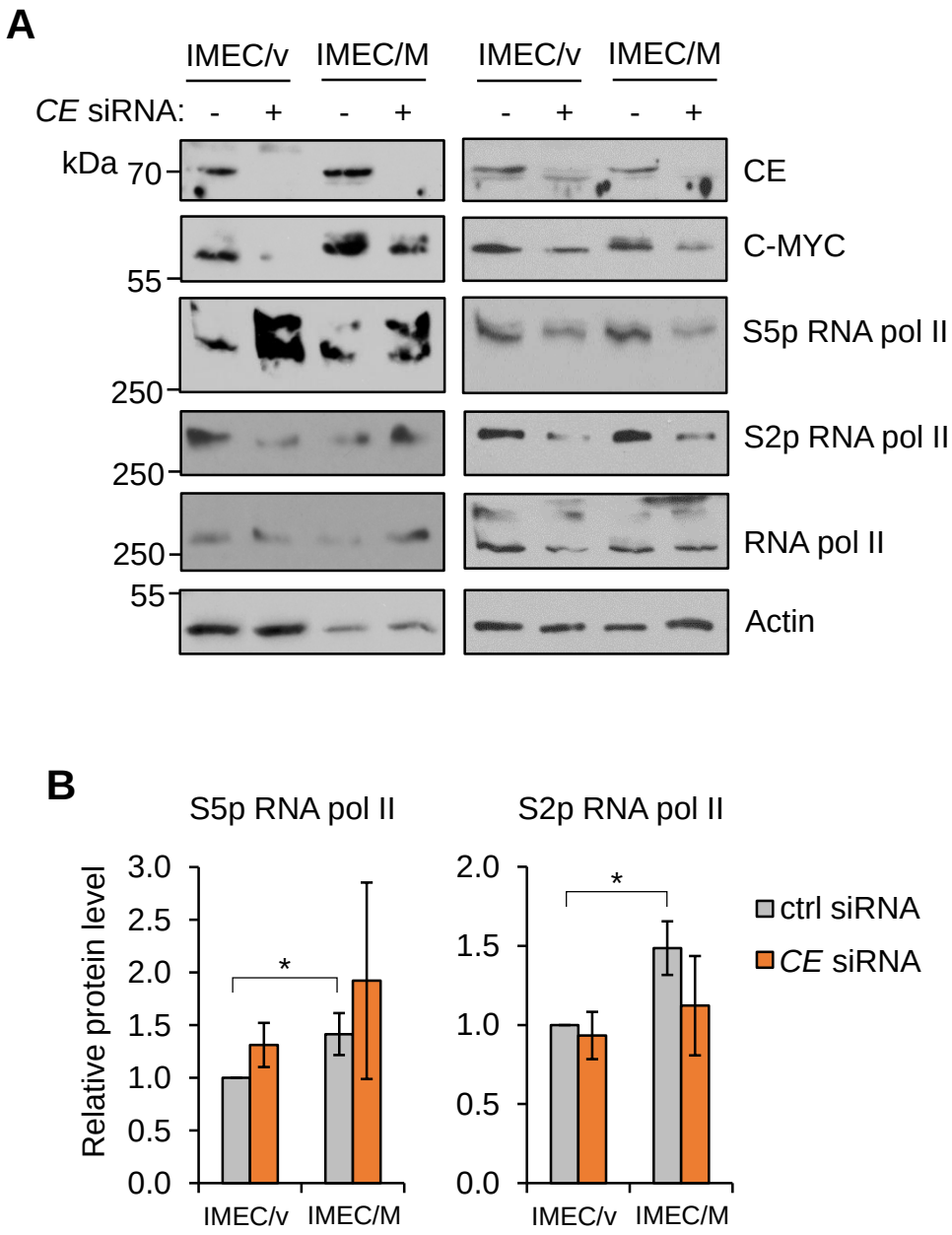

Figure 4.6: CE knockdown does not significantly affect RNA pol II S5 and S2 phosphorylation in IMECs. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting. Two biological replicates are shown. (B) Densitometry was performed using ImageJ software to quantify the S5p or S2p RNA pol II Western blot signal (normalised to pan RNA pol II) following transfection of *CE* siRNA 1 or non targeting control (ctrl). Error bars represent standard error of the mean, n≥4. Significance calculated by Student's t-test, *p≤0.05.

CE inhibition on RNA pol II phosphorylation, the endogenous *CE* locus could be replaced by *CE* fused to an auxin-inducible degron tag or GFP tag using the clustered regularly interspaced short palindromic repeats (CRISPR) system. By addition of auxin, or stable transfection of inducible von Hippel-Lindau E3 ubiquitin ligase fused to a GFP nanobody (respectively), this could induce targeted proteolysis of endogenous CE-GFP in a rapid manner, thus minimising secondary effects of CE inhibition (Fulcher et al., 2016; Natsume et al., 2016).

To further examine the relationship between CE and C-MYC expression, CE was overexpressed to determine if this increased C-MYC levels. In IMECs, stably expressing CE-GFP did not elevate C-MYC expression (Figures 3.4 A and 4.4). Similarly, inducing FLAG-CE overexpression in HeLa cells expressing *FLAG-CE* under control of an inducible promoter did not increase C-MYC expression or RNA pol II phosphorylation (Figure 4.7). In the previous chapter, it was shown that a small fraction of RNA pol II co-purified with CE from cells, consistent with CE being present in excess. This would explain why increasing CE expression had no effect on C-MYC levels.

It was then investigated whether CE knockdown affected C-MYC protein levels in HeLa cells. Consistent with *C-MYC* mRNA levels (Figure 4.5), CE depletion caused reduced C-MYC protein expression, and a concurrent reduction in RNA pol II phosphorylation (Figure 4.8). During the time-course of CE knockdown using two independent siRNAs, CE levels correlate with that of C-MYC, suggesting that CE directly regulates C-MYC expression. Although RNA pol II phosphorylation defects might occur due to reduced C-MYC expression, it is possible that CE influences RNA pol II phosphorylation in other ways. Stably expressing CE-GFP WBL in HeLa cells rescued the defect in

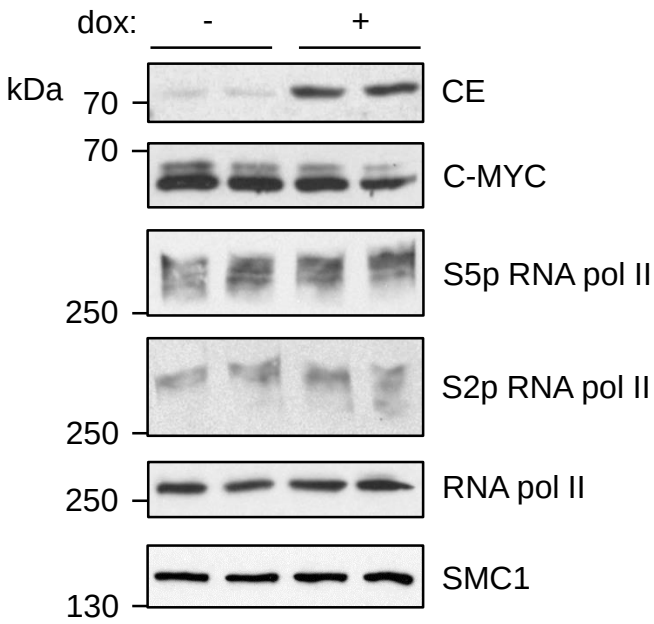

Figure 4.7: Overexpressing CE does not increase C-MYC expression in HeLa cells. HeLa cells stably expressing *FLAG-CE* under control of a tetracycline-inducible promoter were treated with 1µg/ml doxycycline (dox) or DMSO as a control (-). After 48 hours, protein was extracted and analysed by Western blotting. Technical duplicates for this experiment are shown. The cell line was made by Dr. Francisco Inesta-Vaquera in the Cowling lab. Individual experiment.

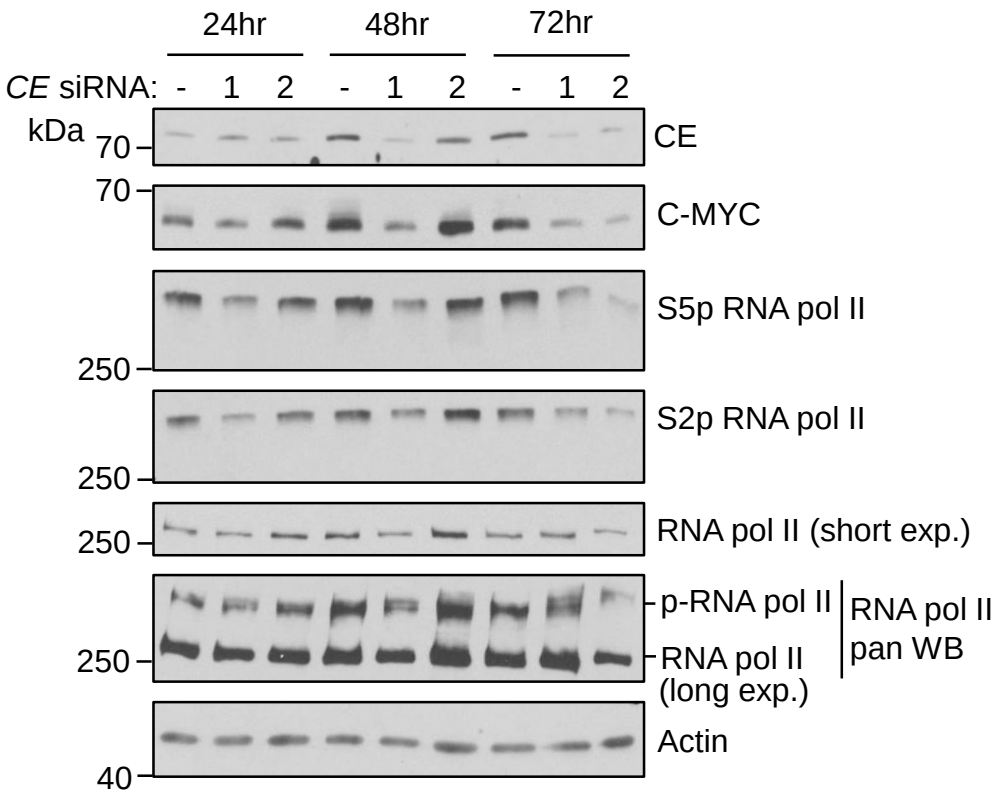

Figure 4.8: Temporal dynamics of CE knockdown, C-MYC levels and RNA pol II phosphorylation. HeLa cells were transfected with 50nM of non-targeting control (ctrl), *CE* siRNA 1 or *CE* siRNA 2. 24, 48 or 72 hours later, protein was extracted and analysed by SDS-PAGE and Western blotting. Individual experiment.

C-MYC expression and S5p RNA pol II phosphorylation in response to CE depletion, confirming these effects are specific (Figure 4.9 A and B).

In summary, CE is required for optimal C-MYC expression in IMECs and HeLa cells. It would be interesting to determine whether this is also true in other cell types.

4.2.2 CE knockdown increases RNA pol II occupancy at the *C-MYC* pause site

To determine if CE depletion in HeLa cells impacted transcription, RNA pol II ChIP was performed. CE has the potential to impact RNA pol II transcription directly, or indirectly via influencing C-MYC expression and RNA pol II phosphorylation. The *C-MYC* gene itself is regulated by RNA pol II pausing, thus primers were designed spanning the *C-MYC* gene to analyse RNA pol II distribution (Figure 4.10 A). This could also indicate whether CE influences C-MYC expression via a transcriptional mechanism. RNA pol II pausing on the *C-MYC* gene occurs near the first exon-intron boundary (Bentley and Groudine, 1988; Keene et al., 1999; Krumm et al., 1992; Spencer and Kilvert, 1993; Strobl and Eick, 1992). Interestingly, CE depletion increased RNA pol II occupancy at the pause site (Figure 4.10 B). This indicates that CE may have a role in RNA pol II pause release. However, there was also a reproducible – although not significant – increase in RNA pol II occupancy at the mid-gene region (+3600) which could indicate a general RNA pol II processivity impairment. To determine is CE knockdown caused a similar effect on other genes, promoter proximal regions of *CCND1* (a C-MYC-repressed gene), *TIP49* (a C-MYC-induced gene) and *RNMT* (not regulated by C-MYC)

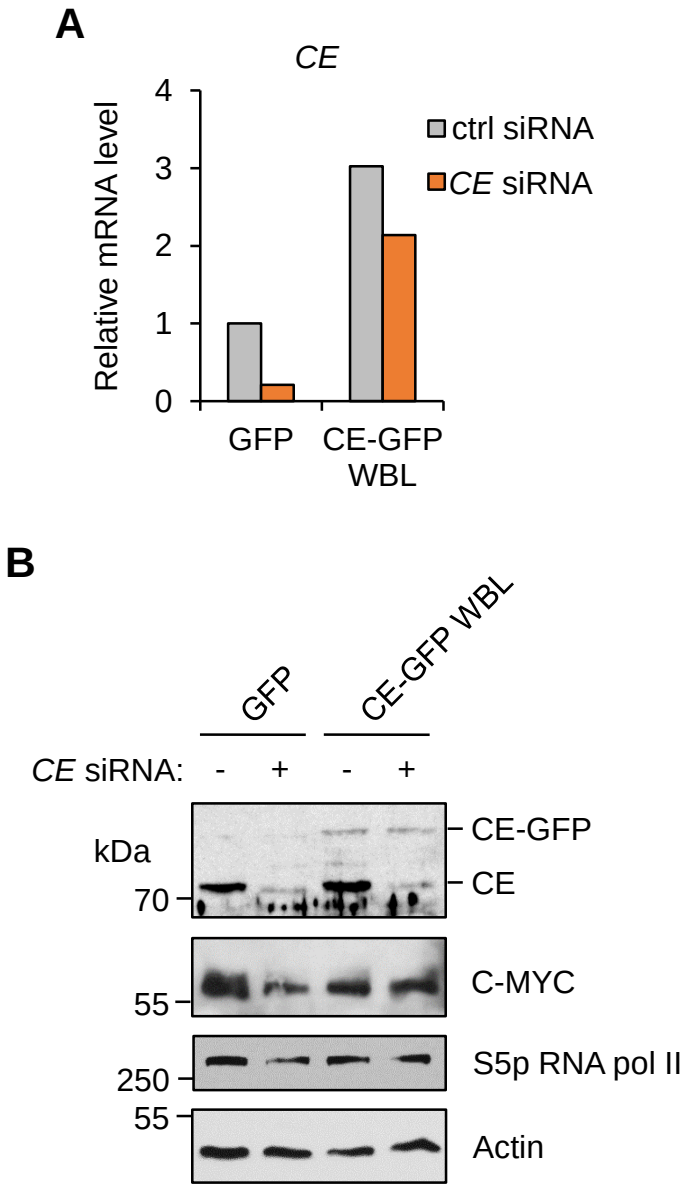

Figure 4.9: siRNA-resistant CE-GFP rescues the C-MYC expression defect upon CE knockdown in HeLa cells. (A) HeLa cells were stably transduced with a construct encoding siRNA-resistant CE-GFP (CE-GFP WBL) or GFP as a control. Cells were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (-). After 72 hours, RNA was extracted from cells and analysed by RT-qPCR. Average of two independent experiments. (B) As in (A) except protein was extracted from cells after 72 hours and analysed by Western blotting. Representative of two independent experiments.

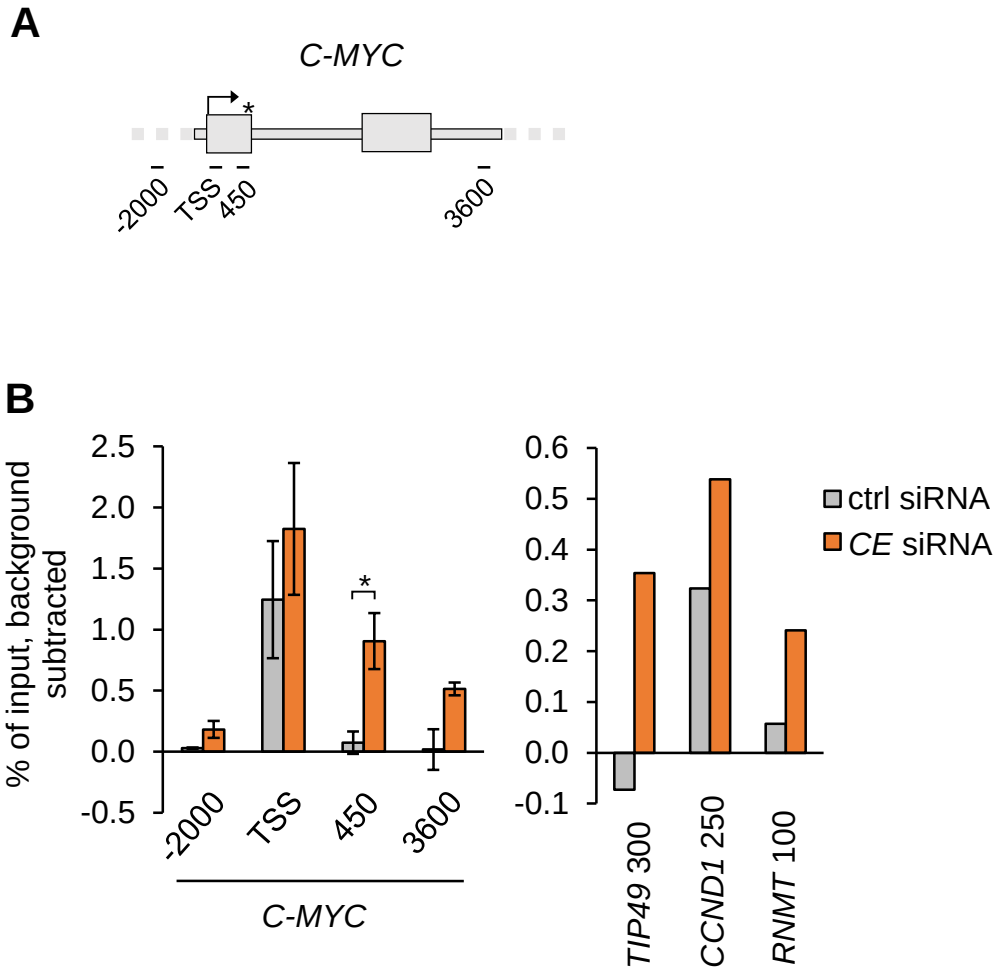

Figure 4.10: CE knockdown increases RNA pol II occupancy on the *C-MYC* gene. (A) Schematic depicting regions of primer amplification used for analysis of RNA pol II ChIP DNA relative to the TSS (arrow) on the *C-MYC* gene. Note that only half of the gene is shown. *RNA pol II pause site. (B) HeLa cells were transfected with 50nM *CE* siRNA 1 or non-targeting control siRNA (ctrl). After 48 hours, RNA pol II was immunoprecipitated and the co-purified DNA analysed by qPCR. Signal is expressed as the RNA pol II-bound DNA relative to DNA in the input. The background signal (in the control ChIP with no antibody) was subtracted. Values indicate position relative to the TSS. Error bars represent standard error of the mean, n=3 for *C-MYC* gene regions. RNA pol II ChIP signal values for other genes is averaged from two independent experiments. Significance was calculated by Student's t-test; *p≤0.05.

were analysed (Figure 4.10 B). These preliminary results indicate that CE may also influence RNA pol II transcription on other genes, although further verification is required. Analysing RNA pol II distribution across these genes (or performing ChIP sequencing) would clarify these observations. Additionally, comparing levels of nascent (unspliced) *C-MYC* mRNA relative to mature (spliced) *C-MYC* mRNA would confirm whether CE influences *C-MYC* transcription. *C-MYC* mRNA synthesis could also be measured using nuclear run-on, during which nascent RNA is synthesised in cells whilst being labelled with bromouridine and then purified for RT-qPCR analysis (or sequencing for global analysis).

4.2.3 Investigating whether CE regulates *C-MYC* expression via the coding region determinant of stability

As previously mentioned, exogenous *FLAG-C-MYC* in IMEC/C-MYC (not expressed from the endogenous *C-MYC* promoter) is sensitive to CE depletion in addition to endogenous *C-MYC* in IMEC/vec and HeLa cells. This indicates that CE mayo regulate *C-MYC* mRNA stability rather than *C-MYC* transcription in IMECs. *C-MYC* mRNA degradation is mediated by two sequences: a region in the 3' UTR and a region at the 3' end of the coding sequence within the third exon called the coding region determinant (CRD) (Brewer and Ross, 1988; Herrick and Ross, 1994; Wisdom and Lee, 1991). The 3' UTR is not present in exogenously expressed *FLAG-C-MYC,* therefore mechanisms involving the CRD may render *C-MYC* mRNA sensitive to CE depletion. The CRD mediates co-translational degradation of *C-MYC* mRNA. A series of rare codons precede the CRD, causing translation to slow down (when availability of the corresponding tRNAs is limiting) and thus the mRNA is subject to degradation

by polysome-associated endonuclease(s) (Lee et al., 1998; Lemm and Ross, 2002). A CRD-binding protein (CRD-BP) stabilises *C-MYC* mRNA by protecting it from endonucleolytic attack (Bernstein et al., 1992; Sparanese and Lee, 2007). It is possible that CE could regulate CRD-BP expression, thus indirectly regulating *C-MYC* mRNA stability. CE was depleted by siRNA in HeLa cells to test this (Figure 4.11). Preliminary data indicates that CRD-BP expression is modestly impacted by CE depletion. However, this is obscured by the fact that CRD-BP is itself a C-MYC target gene (Noubissi et al., 2010), as confirmed by C-MYC knockdown in this system (Figure 4.11). Therefore CE may regulate CRD-BP directly or through C-MYC. Further studies are required to determine if the CRD has a role in CE-dependent *C-MYC* stability. For example, CRD-BP exists in a multimeric complex with four obligate binding partners (Weidensdorfer et al., 2009), any of which might be regulated by CE. Alternatively, perhaps 5'-3' looping of mRNA allows the cap or cap-binding proteins to interact with the CRD or CRD-BP, which could mask the CRD or stabilise CRD-BP binding. It would be interesting to express a mutant *C-MYC* lacking the CRD sequence in cells to determine if this renders *C-MYC* resistant to CE depletion. Importantly, to truly determine if CE influences *C-MYC* mRNA stability, a transcription inhibitor should be used in cells and *C-MYC* transcript decay tracked in the presence and absence of CE knockdown. It would also be interesting to determine whether the *C-MYC* transcript exhibits reduced 7-methylguanosine capping upon CE knockdown using an antibody which specifically detects this cap modification (Cole and Cowling, 2009a).

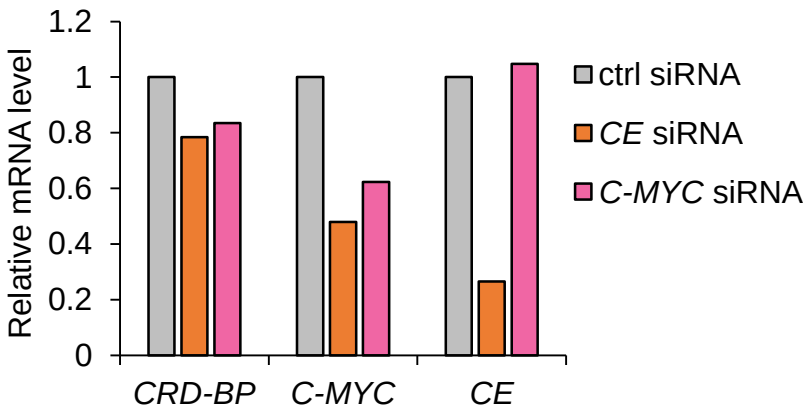

Figure 4.11: Investigating if CE regulates *CRD-BP* expression. HeLa cells were transfected with 50nM *CE* siRNA 1, *C-MYC* siRNA or non-targeting control siRNA (ctrl). After 72 hours, RNA was extracted and analysed by qRT-PCR. Transcript level is normalised to that of *GAPDH*. Individual experiment. This experiment was performed in collaboration with Maria Pisliakova in the Cowling lab.

4.2.4 Investigating if CE influences C-MYC protein stability

Since CE depletion affects *C-MYC* transcript levels, it was suspected that CE primarily influences *C-MYC* mRNA synthesis or stability. However, it is possible that multiple mechanisms are involved, for example CE influencing *C-MYC* translation or indirectly impacting C-MYC protein stability. To test the latter possibility, HeLa cells were treated with the proteasome inhibitor carbobenzoxy-Leu-Leu-leucinal (MG132) to prevent proteasomal degradation of C-MYC. As expected, since C-MYC has a high turnover rate, C-MYC protein generally accumulated in a dose- and time- dependent manner in response to MG132 treatment (Figure 4.12 A). Preliminary experiments showed that when C-MYC degradation was inhibited, there was still a relative decrease in C-MYC protein levels in response to CE knockdown (Figure 4.12 B), indicating that CE does not significantly affect C-MYC protein stability in HeLa cells.

4.2.5 C-MYC overexpression sensitises C-MYC target genes to CE depletion

It was then investigated whether CE regulates C-MYC target genes. CE was knocked down in IMEC/vec and IMEC/C-MYC before analysing C-MYC target gene mRNA levels. This experiment probes the net effect of CE on C-MYC target genes which encompasses several potential mechanisms: 1) CE directly regulating C-MYC target genes; 2) CE controlling C-MYC expression; and 3) other secondary effects of CE knockdown. *NCL*, *ODC*, *NME1*, *NPM*, *FBL* and *TIP49* were verified as C-MYC-induced genes in IMECs (Figure 4.13 A). CE depletion reversed the induction of these target genes in IMEC/C-MYC (Figure 4.13 A). Notably, C-MYC-induced genes were unaffected by CE

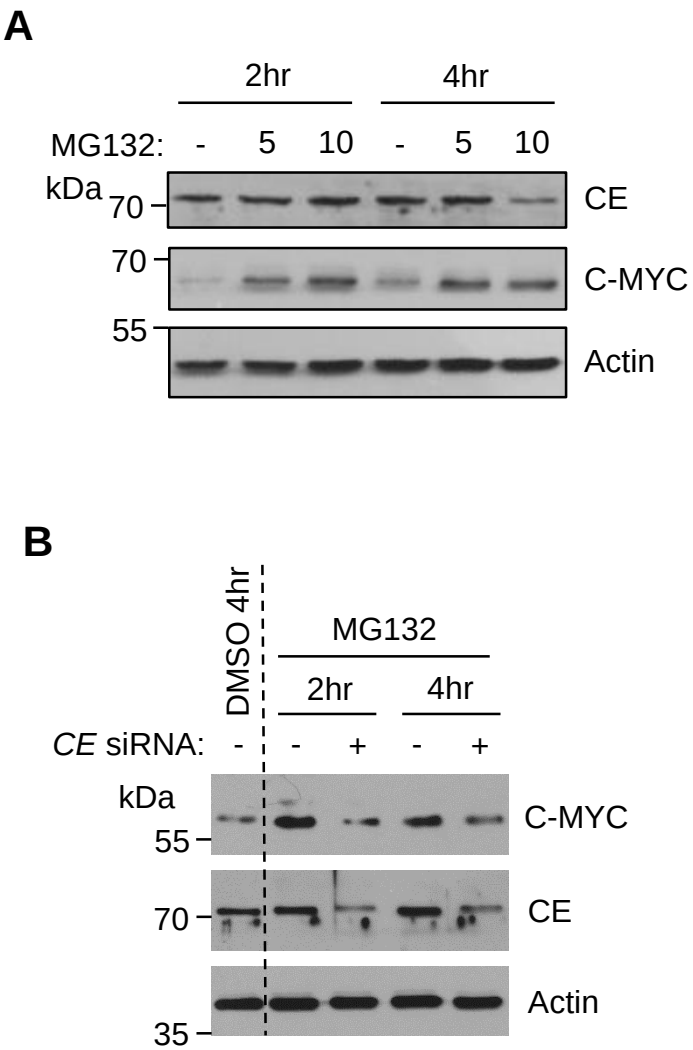

Figure 4.12: Investigating if CE influences C-MYC protein stability. (A) HeLa cells were treated with DMSO (-) or MG132 (5μM or 10μM) for the indicated times before protein was extracted from cells and analysed by Western blotting. Individual experiment. (B) HeLa cells were transfected with 50nM CE siRNA 1 (+) or non-targeting control siRNA (-). After 72 hours, cells were treated with DMSO or 5μM MG132 for the indicated times before protein was extracted from cells and analysed by Western blotting. Individual experiment. These experiments were performed in collaboration with Olga Suska and Maria Pisliakova in the Cowling lab.

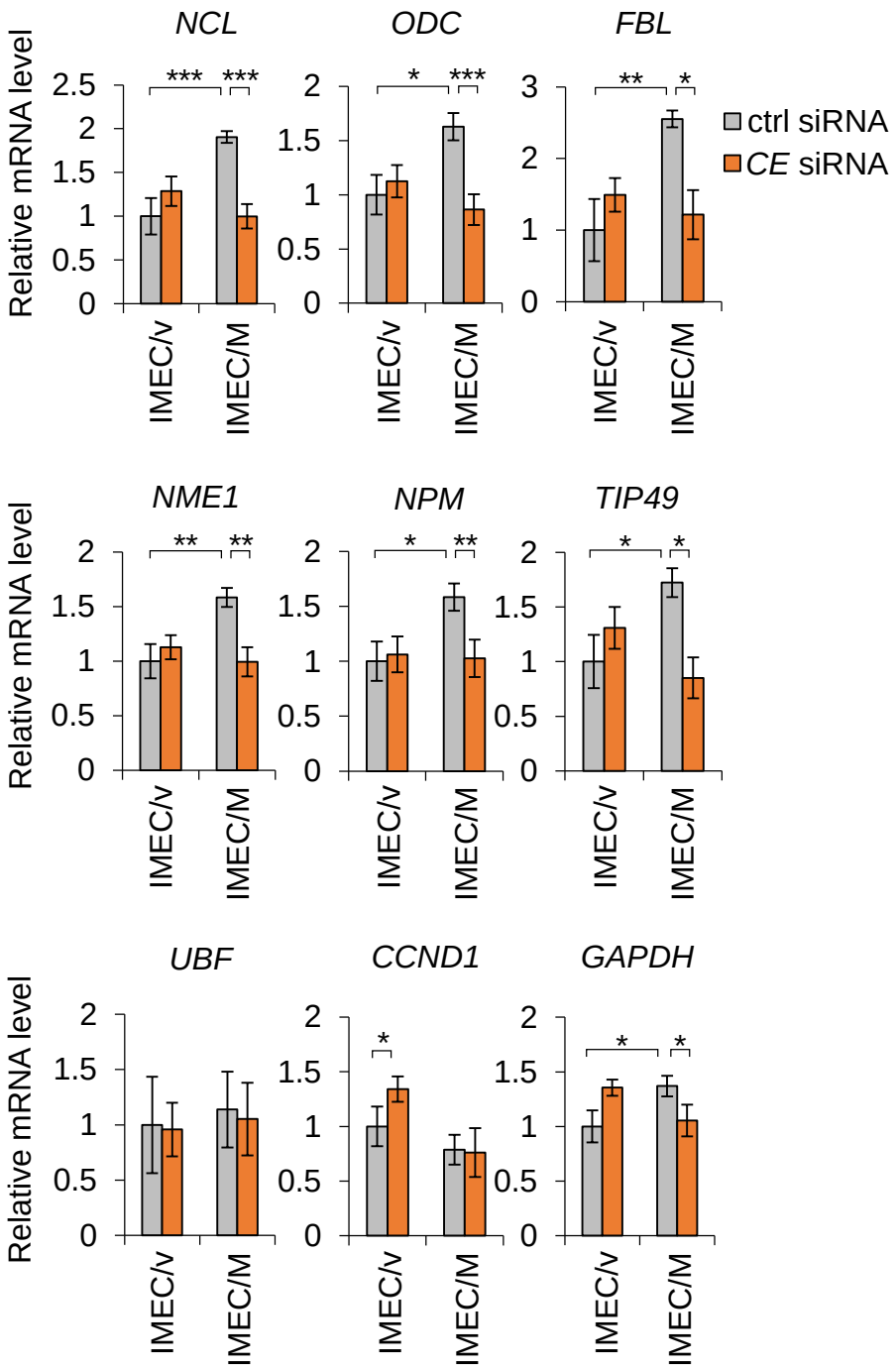

Figure 4.13: C-MYC overexpression sensitises C-MYC target genes to CE depletion. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with CE siRNA 1 or a non-targeting control (ctrl) siRNA. RNA was extracted after 72 hours and analysed by RT-qPCR. qPCR data was not normalised since the reference gene (GAPDH) was significantly changing. Error bars represent standard error of the mean, n≥3. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01, ***≤0.001. Figure continued overleaf.

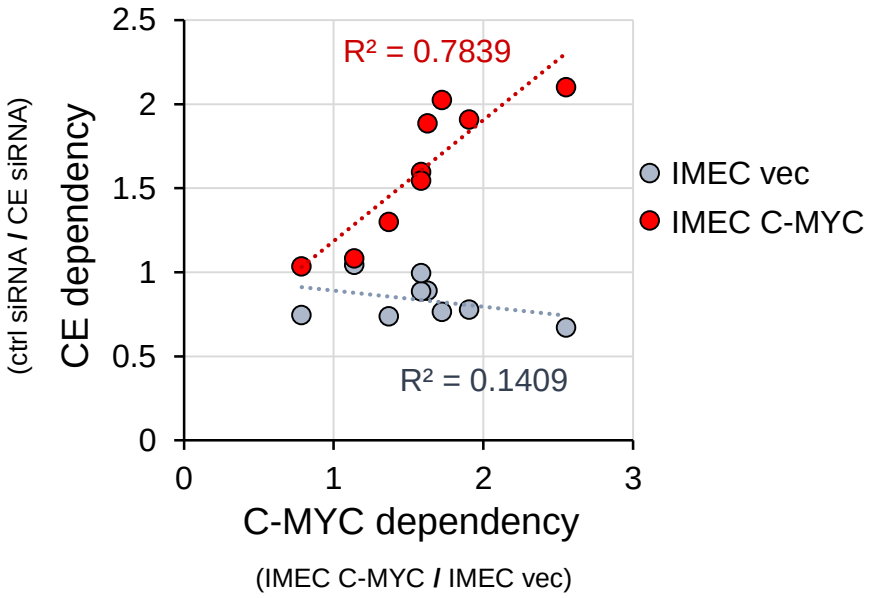

Figure 4.13 continued: C-MYC overexpression sensitises C-MYC target genes to CE depletion. (B) For each gene analysed in part (A), the fold-change in mRNA level upon CE knockdown (ctrl siRNA / CE siRNA, i.e. CE dependency) is plotted against the fold-change in mRNA level upon C-MYC overexpression (IMEC vec / IMEC C-MYC, i.e. C-MYC dependency).

depletion in IMEC/vec, which only express endogenous C-MYC (Figure 4.13 A). This was surprising given that endogenous C-MYC expression is impaired by CE knockdown. *GAPDH* was modestly induced by C-MYC and modestly depleted by CE knockdown (Figure 4.13 A). This indicates that the CE and C-MYC interplay may act globally, albeit to a lesser extent than with canonical C-MYC target genes. More comprehensive studies are required to confirm this. As previously mentioned, canonical and non-canonical E-boxes reside 300-800 bases from the *GAPDH* TSS which might explain why it is C-MYC-responsive. *UBF* (upstream binding factor), a C-MYC target gene in some systems (Poortinga et al., 2004a; Wall et al., 2008), was unresponsive to both C-MYC and CE knockdown in IMECs. Transcript levels of *CCND1* – a C-MYC-repressed gene in IMECs (Cowling, 2009; Philipp et al., 1994) (Figure 4.16 C) – slightly increased when CE was depleted in IMEC/vec (Figure 4.13), correlating with reduced C-MYC expression. However, when *CCND1* was repressed by elevated C-MYC expression, it was unresponsive to CE depletion. This could be because C-MYC levels in IMEC/C-MYC/*CE* siRNA cells were generally 4-fold higher than in IMEC/vec/control siRNA cells (Figure 4.2 B), indicating that sufficient C-MYC remained to maintain *CCND1* repression. Taken together, there is a good correlation between gene dependency on CE and gene dependency on C-MYC, but only in the presence of C-MYC deregulation (Figure 4.13 B). This suggests that CE may preferentially regulate genes targeted by overexpressed C-MYC rather than endogenous C-MYC, and that CE inhibition may have a biased effect on these genes in cells with deregulated C-MYC. Expression of siRNA-resistant CE-GFP rescued the defect in C-MYC target gene expression in IMEC/C-MYC in response to CE suppression (Figure 4.14 A, individual genes and Figure 4.14 B, average results for 6 genes),

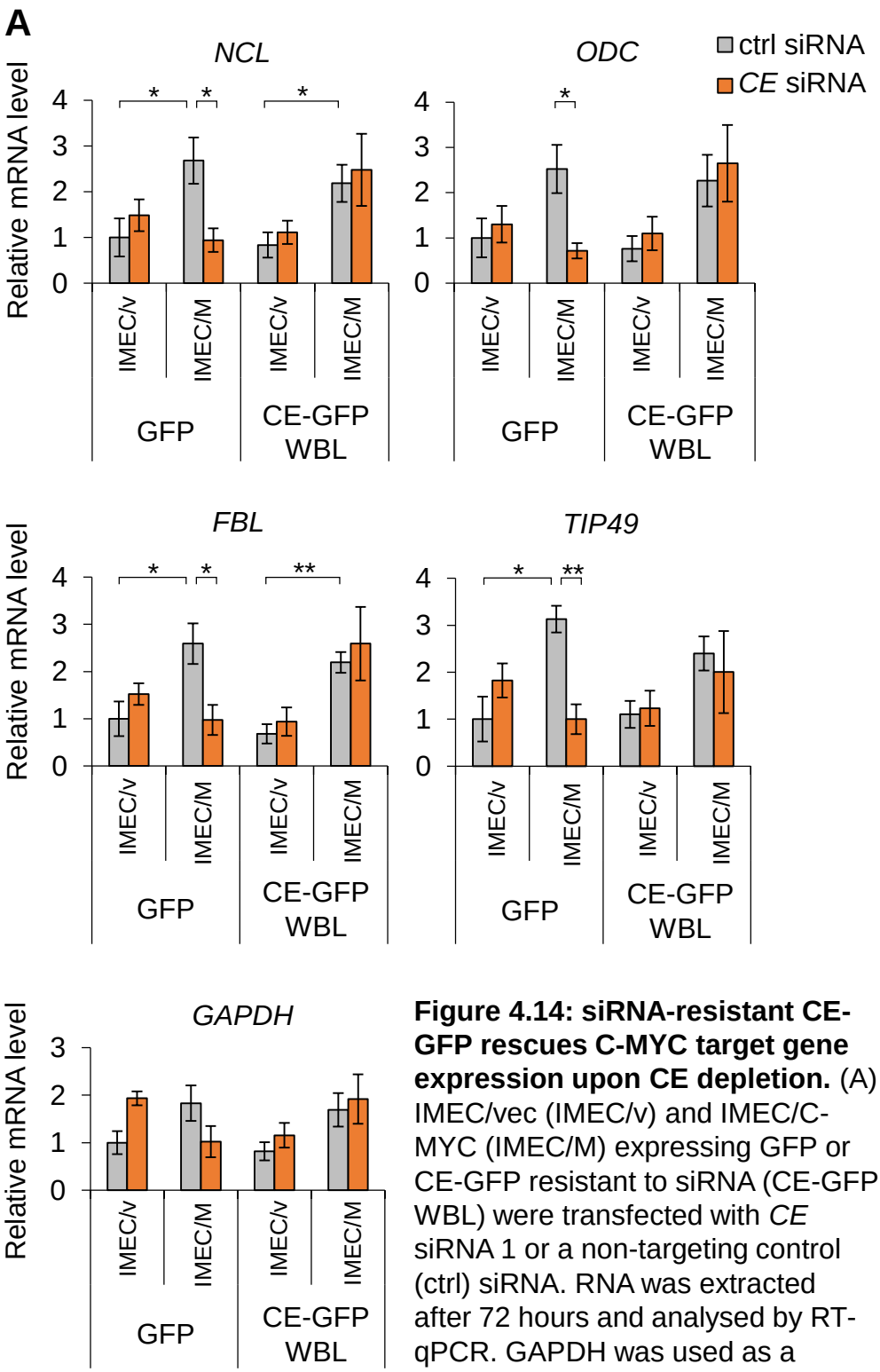

Figure 4.14: siRNA-resistant CE-GFP rescues C-MYC target gene expression upon CE depletion. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) expressing GFP or CE-GFP resistant to siRNA (CE-GFP WBL) were transfected with *CE* siRNA 1 or a non-targeting control (ctrl) siRNA. RNA was extracted after 72 hours and analysed by RT-qPCR. GAPDH was used as a reference gene but qPCR data is not normalised. Error bars represent standard error of the mean, n=3. Significance was calculated by Student's t-test; *p≤0.05, **p≤0.01, ***≤0.001. Figure continued overleaf.

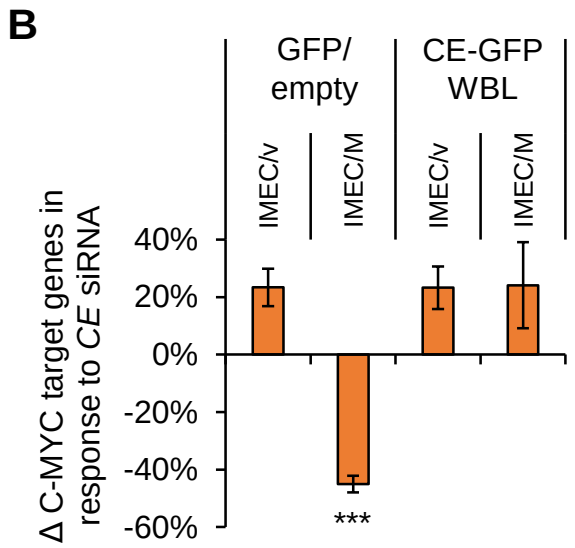

Figure 4.14 continued: siRNA-resistant CE-GFP rescues C-MYC target gene expression upon CE depletion. (B) Average change (Δ) in mRNA levels of C-MYC target genes (NCL, ODC, FBL, NME1, NPM and TIP49) upon CE depletion relative to control transfections. Error bars represent standard error of the mean, n=6. Significance was calculated by Student's t-test; ***≤0.001.

indicating that the effect of CE knockdown on C-MYC target genes is specific. To confirm that these trends are reflected at the protein level, NCL protein expression was analysed in IMECs upon CE knockdown (Figure 4.15 A and B). Consistent with mRNA levels, NCL protein was diminished by CE depletion in IMEC/C-MYC, but was not affected in IMEC/vec. In summary, IMECs with overexpressed C-MYC are dependent on CE for inducing C-MYC target genes above the basal level, whereas their expression in IMECs with normal, endogenous C-MYC levels is not CE-dependent.

4.2.6 Investigating the relationship between low *C-MYC* levels and resistance of C-MYC target genes to CE depletion

The observation that C-MYC target gene expression was unaffected by CE depletion in IMEC/vec, despite endogenous C-MYC levels being reduced, was unexpected. With the premise that C-MYC and N-MYC share a substantial proportion of target genes and are partially functionally redundant, in addition to the fact that *N-MYC* is a C-MYC-repressed gene (Breit and Schwab, 1989; Malynn et al., 2000; Westermann et al., 2008), it was tested if N-MYC compensated for C-MYC loss in IMEC/vec (Figure 4.16 A). N-MYC was undetectable in IMEC/vec, and only trace amounts were detected in IMEC/C-MYC. Alternatively, it was possible that the genes analysed are only regulated by C-MYC when C-MYC is expressed above a certain threshold. To investigate this, IMEC/vec were transfected with *C-MYC* siRNA. Endogenous C-MYC levels were diminished to a very low level (Figure 4.16 B). RNA pol II S5 phosphorylation decreased in response to C-MYC suppression, indicating that endogenous C-MYC is functional in IMEC/vec (Figure 4.16 B). *NCL*, *ODC* and *TIP49* transcripts were insensitive to C-MYC knockdown, whereas *NME1*, *NPM*

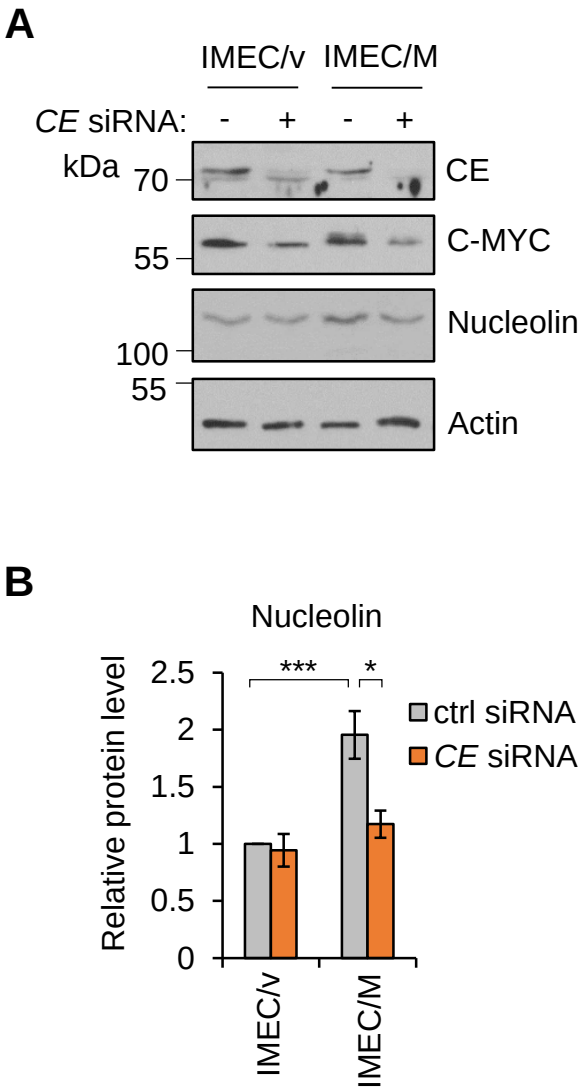

Figure 4.15: The sensitivity of C-MYC target genes to CE depletion is reflected at the protein level. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with *CE* siRNA 1 or a non-targeting control siRNA (-). Protein was extracted after 72 hours and analysed by Western blotting. (B) Densitometry was performed using ImageJ software to quantify the nucleolin Western blot signal (normalised to actin) following transfection of CE siRNA or non-targeting control (ctrl) as above. Error bars represent standard error of the mean, n≥3. Significance was calculated by Student's t-test; *p≤0.05, ***≤0.001.

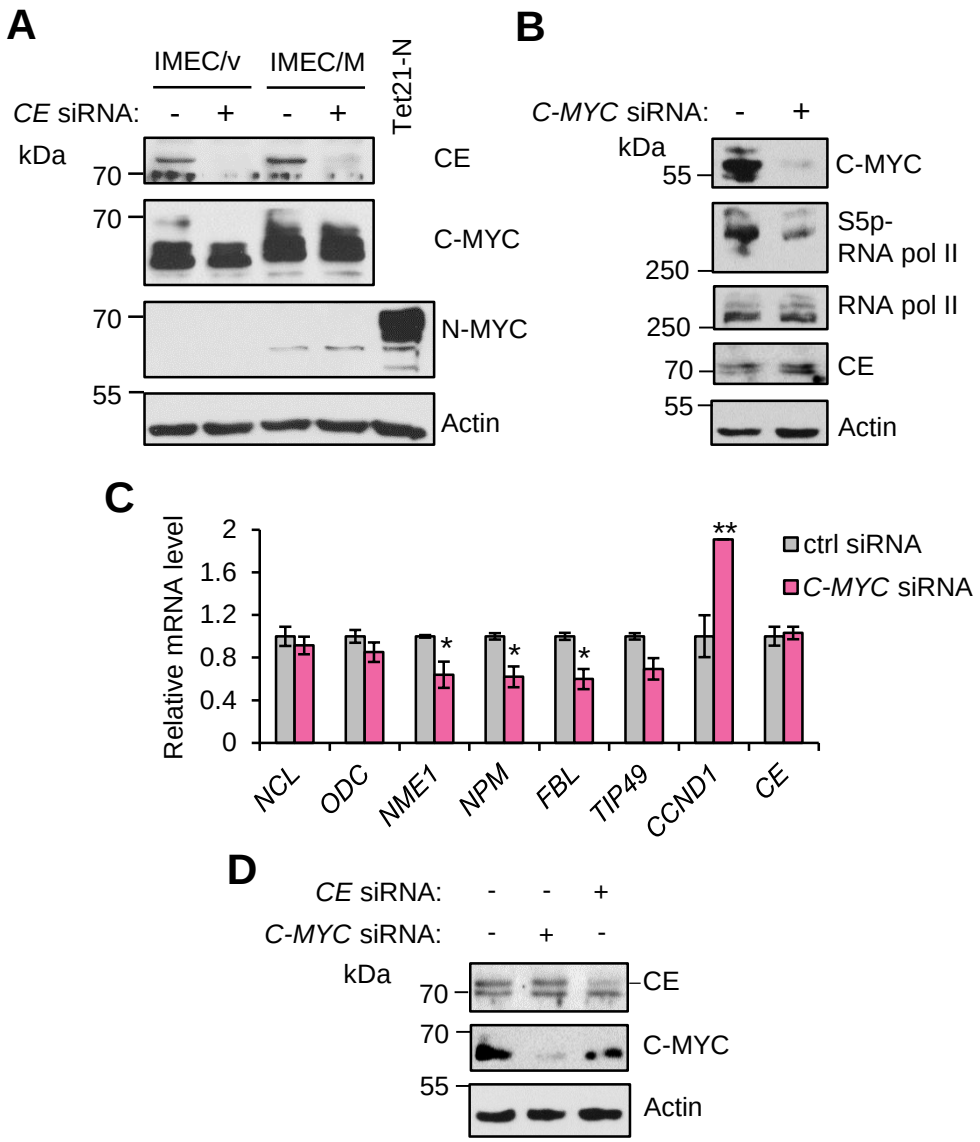

Figure 4.16: Investigating the resistance of C-MYC target genes in IMECs to CE depletion. (A) IMEC/vec (IMEC/v) and IMEC/C-MYC (IMEC/M) were transfected with 50nM *CE* siRNA 1 or a non-targeting control (-). After 72 hours, protein was extracted and analysed by Western blotting. Tet21-N cell protein was used as a positive control for N-MYC expression. Individual experiment. (B) IMEC/vec were transfected with *C-MYC* siRNA or non-targeting control (-). After 72 hours, protein was extracted and analysed by Western blotting. Representative of three independent experiments. (C) As in (B) but RNA was extracted and analysed by RT-qPCR. Transcript levels are expressed relative to that of GAPDH. Error bars represent standard error of the mean, n=6. Significance calculated by Student's t-test, **p≤0.01; *p≤0.05. (D) IMEC/vec were transfected with *C-MYC* siRNA, *CE* siRNA 1 or non-targeting control (-). After 72 hours, protein was extracted and analysed by Western blotting. Representative of two independent experiments.

and *FBL* transcript levels were sensitive (Figure 4.16 C). It should be noted that *C-MYC* siRNA caused a greater decrease in C-MYC expression than *CE* siRNA (Figure 4.16 D). Therefore, *NCL*, *ODC* and *TIP49* are not regulated by endogenous C-MYC in IMEC/vec, which could explain why they are resistant to the effects of CE depletion. On the other hand, *NME1*, *NPM* and *FBL* are target genes of low level endogenous C-MYC, but are resistant to the intermediate level of C-MYC depletion observed upon CE knockdown.

4.2.7 Exploring a method to functionally uncouple CE regulating C-MYC and C-MYC target genes

As stated above, overexpressed C-MYC requires CE for inducing expression of its target genes. However, it is not clear whether this dependency is due to the requirement of CE to maintain C-MYC expression, if C-MYC requires CE as a transcriptional co-factor to directly regulate its target genes, or if overexpressed C-MYC induces CE dependency through other means. In an attempt to functionally uncouple CE regulating C-MYC expression and function, it was investigated if there was a temporal window during the process of CE knockdown when CE was depleted before C-MYC protein levels were affected. Since *C-MYC* mRNA and protein are both unstable, and C-MYC is very responsive to changes in CE expression (Figure 4.8), IMECs expressing C-MYC T58A (designated IMEC/T58A) were used in order to maximise the time after CE knockdown before C-MYC was affected. This mutation typically stabilises the protein by 2-5-fold, as it impairs C-MYC phospho-dependent ubiquitination and degradation (Gregory and Hann, 2000; Salghetti et al., 1999; Sears et al., 2000). As previously observed, C-MYC T58A protein levels are higher than C-MYC WT (Figure 4.17), consistent with it being stabilised. The

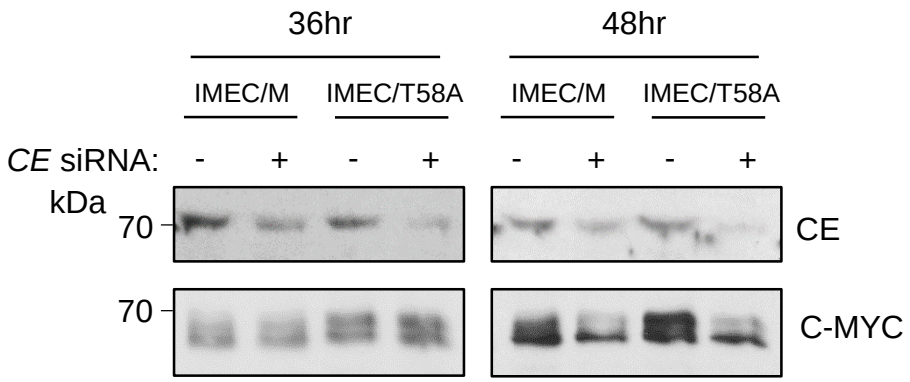

Figure 4.17: Investigating C-MYC T58A stability in response to CE depletion. IMECs stably transduced with the wild-type C-MYC vector (IMEC/M) or a C-MYC vector carrying the T58A mutation (IMEC/T58A) were transfected with *CE* siRNA 1 or a non-targeting control (-). After 36 or 48 hours, protein was extracted and analysed by Western blotting. Individual experiment.

effect of CE knockdown on C-MYC expression in IMEC/C-MYC and IMEC/T58A was compared after 36-48 hours (Figure 4.17). After 36 hours, CE was at least partially depleted whereas neither C-MYC WT nor C-MYC T58A were affected. However, both C-MYC variants were substantially diminished after 48 hours. Following the observation that many C-MYC target genes take over 24 hours to be affected by C-MYC depletion (Figure 3.14 A and B), it seems it would be challenging to dissect how CE regulates C-MYC target genes using this method.

4.2.8 C-MYC suppression in cancer cells desensitises C-MYC target genes to CE depletion

The role of CE in regulating C-MYC target genes was next investigated in cancer cells with deregulated C-MYC. HeLa cells overexpress C-MYC as a result of viral insertion at the *C-MYC* gene, causing strong transcriptional activation (Adey et al., 2013). C-*MYC* siRNA was used to modulate endogenous C-MYC expression in addition to *CE* siRNA. Cells were simultaneously transfected with combinations of the two targeting siRNAs and/or non-targeting control and conditions were optimised (Figure 4.18 A and B; Figure 3.13 A and B). The experiment was designed based upon whether effective knockdown was achieved, if this was equivalent in the presence and absence of the other siRNA, whether C-MYC knockdown was sufficient to reduce S5p RNA pol II levels and whether there would be sufficient time following knockdown to impact C-MYC target gene expression (72 hour knockdown using 25nM *C-MYC* siRNA

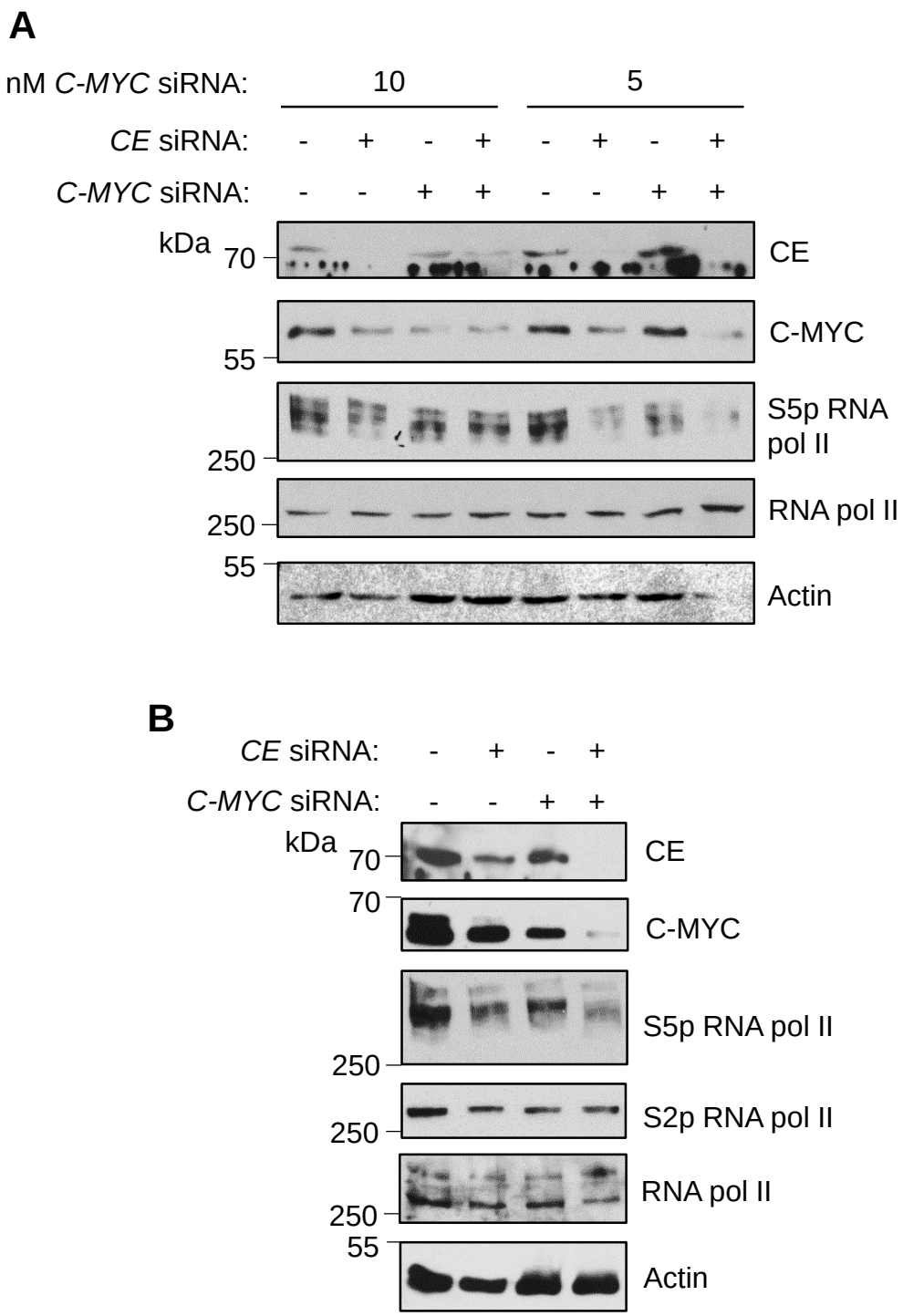

Figure 4.18: Optimisation of C-MYC and CE double knockdown in HeLa cells. (A) HeLa cells were transfected with combinations of *CE* siRNA 1, *C-MYC* siRNA and non-targeting control siRNA (-). 50nM of *CE* siRNA was used and either 10nM or 5nM of *C-MYC* siRNA. After 72 hours, protein was extracted and analysed by Western blotting. Individual experiment. (B) As in (A) except 25nM of *CE* siRNA and 25nM of *C-MYC* siRNA was used. Individual experiment.

and 50nM *CE* siRNA selected, Figure 4.20 A). It should be noted that C-MYC knockdown occurs in less than 24 hours following *C-MYC*-siRNA transfection (Figure 3.13 B) whereas CE depletion was not detected until 48 hours after *CE* siRNA transfection in HeLa cells (Figure 4.8). Therefore, in this experimental arrangement, CE is depleted following changes in C-MYC protein level. *CE* siRNA caused equivalent depletion of *CE* mRNA (Figure 4.19 A) and protein (Figure 4.20 A) in the presence and absence of *C-MYC* siRNA. Strangely, *C-MYC* siRNA was not particularly effective in depleting *C-MYC* mRNA levels (Figure 4.19 A), but was efficient in suppressing C-MYC protein levels (Figures 4.20 A and B); to a similar extent in the presence and absence of *CE* siRNA (Figure 4.20 C). The primary mechanism of silencing by this *C-MYC* siRNA oligo is likely translation repression rather than mRNA degradation. As in IMECs, C-MYC expression was dependent on CE; both *C-MYC* mRNA (Figures 4.19 A and B) and C-MYC protein (Figures 4.20 A and B) were sensitive to CE knockdown, in the presence and absence of *C-MYC* siRNA. Interestingly, *CE* siRNA caused a greater relative reduction in C-MYC levels when C-MYC was knocked down by siRNA (Figure 4.20 D).

CE depletion in HeLa cells resulted in a substantial repression of C-MYC target genes (Figure 4.21 A). On the other hand, when C-MYC was repressed by siRNA, CE depletion had a dampened effect on C-MYC target genes compared to that in HeLa cells with high C-MYC levels (Figure 4.21 A). On average, C-MYC target genes in HeLa cells were significantly (1.5-fold) more sensitive to CE depletion in the presence of deregulated C-MYC (Figure 4.21 B), indicating that C-MYC overexpression heightens the dependency of target

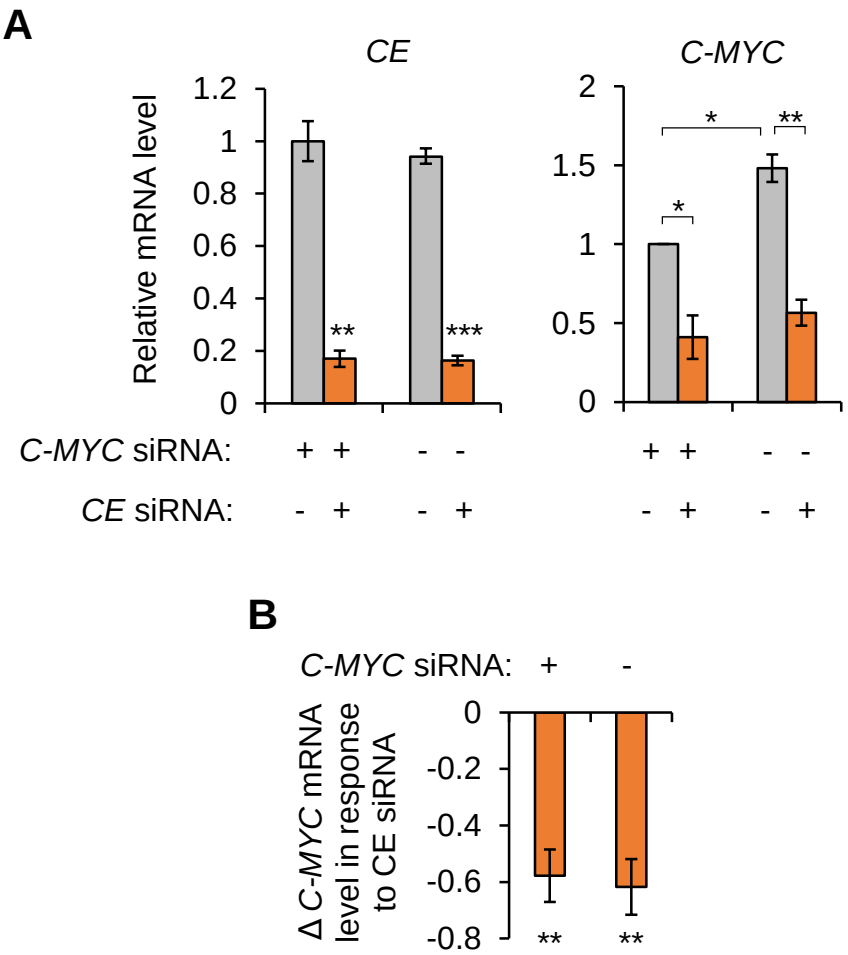

Figure 4.19: Analysis of *CE* and *C-MYC* mRNA levels in single- and double-target knockdowns. (A) HeLa cells were transfected with combinations of *CE* siRNA 1, *C-MYC* siRNA and non-targeting control siRNA (-). 50nM *CE* siRNA and 25nM *C-MYC* siRNA was used. After 72 hours, RNA was extracted and analysed by RT-qPCR. Error bars represent standard error of the mean, n=4. (B) As in (A) but expressed as change (Δ) in *C-MYC* mRNA level following CE depletion in the presence (+) or absence (-) of *C-MYC* siRNA (relative to control transfections). Error bars represent standard error of the mean, n=4. Significance calculated by Student's t-test, *p≤0.05; **p≤0.01; ***≤0.001.

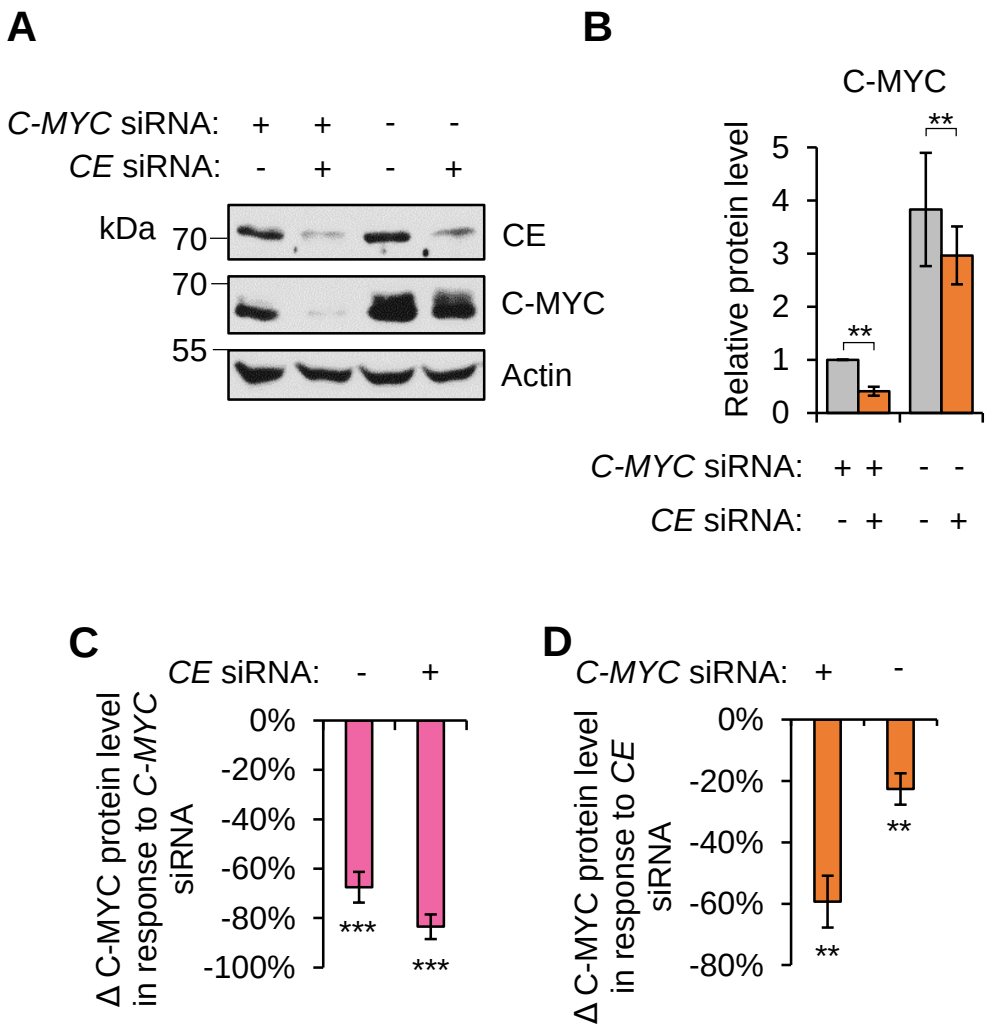

Figure 4.20: Analysis of C-MYC protein expression upon CE and C-MYC double knockdown. (A) HeLa cells were transfected with combinations of *C-MYC* siRNA, *CE* siRNA 1 and non-targeting control siRNA (-). 50nM *CE* siRNA and 25nM *C-MYC* siRNA was used. After 72 hours, protein was extracted and analysed by Western blotting. (B) Densitometry was performed using ImageJ software to quantify the C-MYC Western blot signal (normalised to actin) following transfection of siRNA as above. Error bars represent standard error of the mean, n≥5. (C) As in (B) but expressed as change (Δ) in expression following C-MYC depletion in the presence (+) or absence (-) of *CE* siRNA relative to control transfections. Error bars represent standard error of the mean, n≥5. (D) As in (B) but expressed as change in expression following CE depletion in the presence or absence of C-MYC siRNA relative to control transfections. Error bars represent standard error of the mean, n≥5. Significance calculated by Student's t-test, *p≤0.05; **p≤0.01; ***≤0.001.

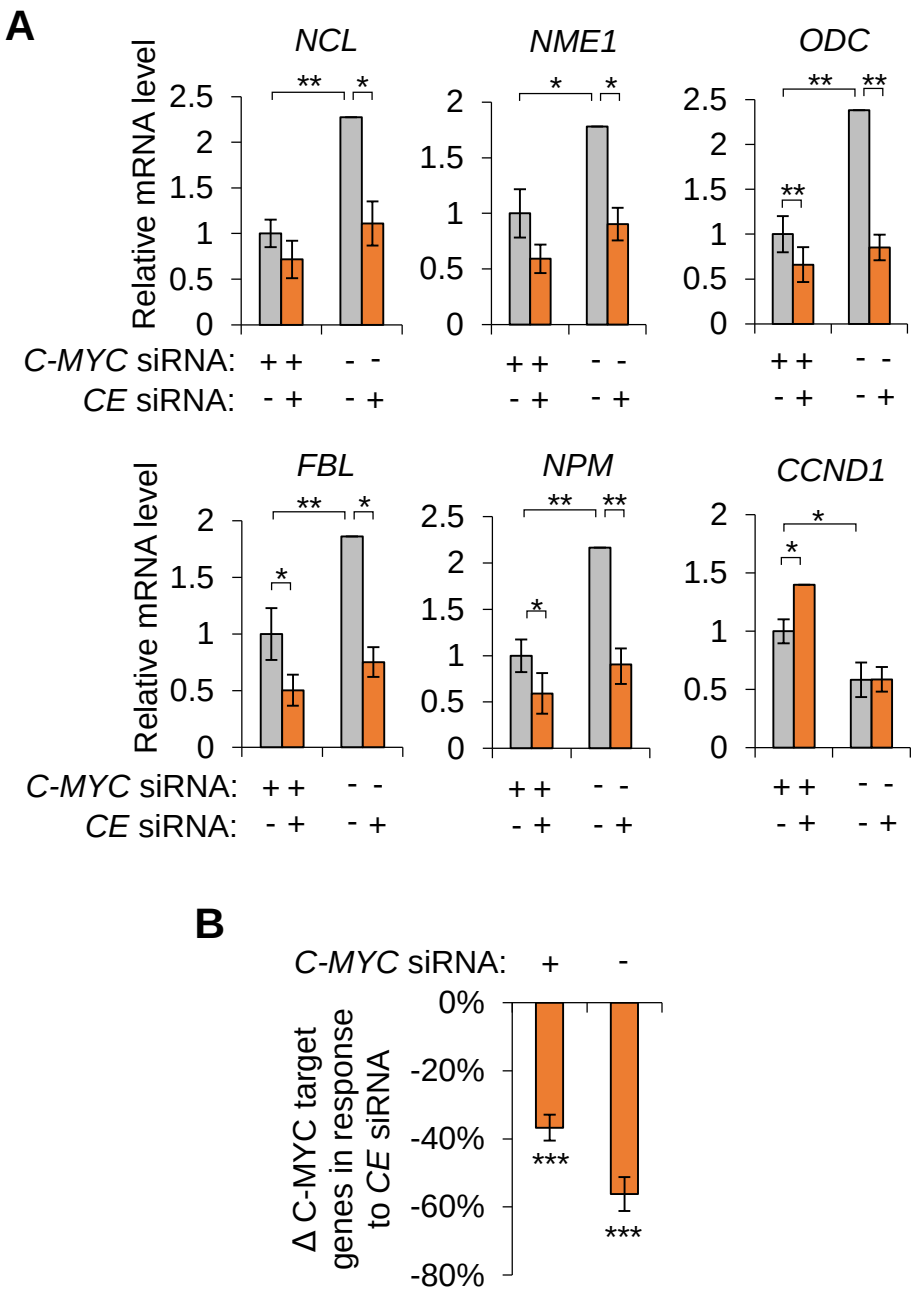

Figure 4.21: C-MYC depletion desensitises C-MYC target genes to CE knockdown. (A) HeLa cells were transfected with combinations of C-MYC siRNA, CE siRNA 1 and non-targeting control siRNA (-). 50nM CE siRNA and 25nM C-MYC siRNA was used. After 72 hours, RNA was extracted and analysed by RT-qPCR. Error bars represent standard error of the mean, n=4. (B) Average change (Δ) in mRNA levels of C-MYC target genes (NCL, NME1, ODC, FBL, and NPM) upon CE depletion relative to control transfections in the presence (+) and absence (-) of C-MYC siRNA. Error bars represent standard error of the mean, n=5. Significance calculated by Student's t-test, *p≤0.05; **p≤0.01; ***≤0.001. Figure continued overleaf.

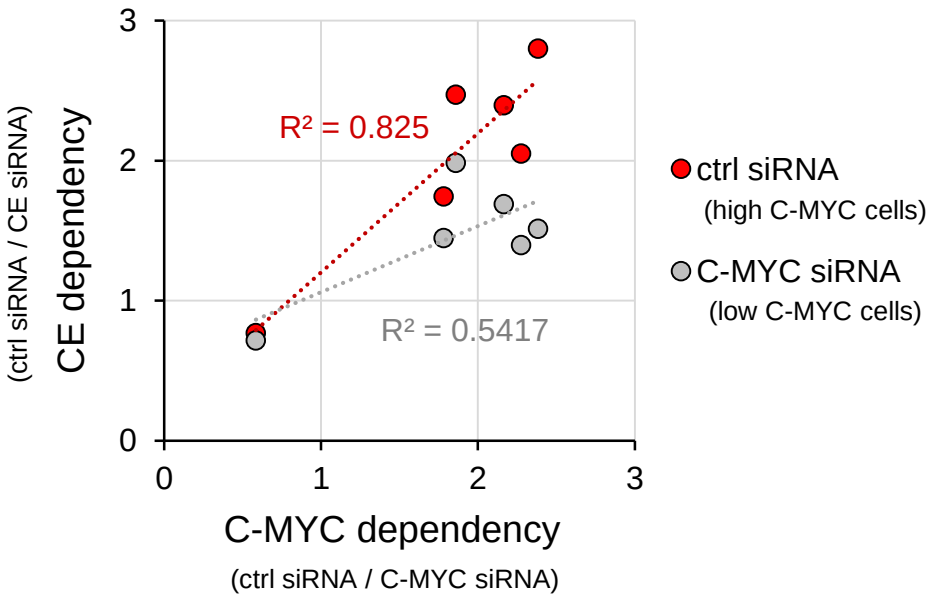

Figure 4.21 continued: C-MYC depletion desensitises C-MYC target genes to CE knockdown. (C) For each gene, fold-change in mRNA level upon CE knockdown (CE dependency) is plotted against the fold change in mRNA level upon C-MYC knockdown (C-MYC dependency).

genes on CE. As in IMECs, the C-MYC-repressed gene *CCND1* was unaffected by CE depletion in cells expressing high levels of C-MYC (Figure 4.21 A), perhaps because sufficient C-MYC remains to maintain *CCND1* suppression (Figure 4.20 B). However, unlike IMECs, no significant changes in *GAPDH* levels were observed upon CE and/or C-MYC knockdown (data not shown). In conclusion, suppression of endogenously overexpressed C-MYC desensitises its target genes to CE depletion. Interestingly, there was a correlation between gene dependency on CE and gene dependency on C-MYC selectively in the presence of C-MYC overexpression (Figure 4.21.C), which is complementary to the above observations in IMECs. This indicates that genes regulated by overexpressed C-MYC may have a heightened dependency on CE for their expression.

4.2.9 CE regulates C-MYC target genes partially independently of regulating C-MYC expression

The above data provide insight into whether CE knockdown only influences C-MYC target gene expression by regulating C-MYC, or whether it directly (or indirectly by other means) regulates C-MYC target genes. C-MYC knockdown in HeLa cells causes a 3-fold greater decrease in C-MYC levels than CE knockdown (Figure 4.22 A). However, CE knockdown diminishes C-MYC target gene expression to the same extent as C-MYC knockdown (Figure 4.22 B, average change in expression of five genes). This indicates that CE does not control C-MYC target genes simply by regulating C-MYC expression. A similar observation can be drawn from IMECs: CE knockdown in IMEC/C-MYC results in net C-MYC levels which are generally 4-fold higher than that in

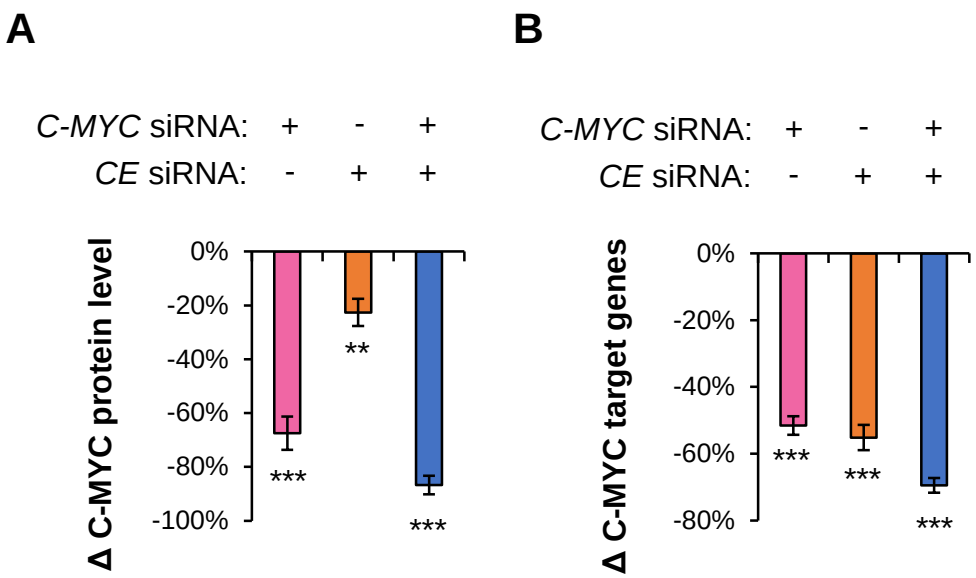

Figure 4.22: CE regulation of C-MYC target genes is partially independent of C-MYC expression. (A) Densitometry was performed using ImageJ software to quantify change (Δ) in C-MYC Western blot signal (normalised to actin) relative to control transfections following C-MYC knockdown, CE knockdown or both. An exemplar Western blot is depicted in Figure 4.21-A. Error bars represent standard error of the mean, n≥5. (B) Average change (Δ) in mRNA levels of C-MYC target genes (NCL, NME1, ODC, FBL, and NPM) upon CE depletion relative to control transfections. Error bars represent standard error of the mean, n=5. Significance calculated by Student's t-test, **p≤0.01; ***≤0.001.

IMEC/vec/control (Figure 4.2 B) and yet C-MYC target gene expression in IMEC/C-MYC is abolished to that in IMEC/vec (Figure 4.13 A). Of note, in IMECs certain C-MYC target genes are insensitive to changes in C-MYC below a certain threshold of C-MYC expression (Figure 4.16 B), which might partially account for this phenomenon. C-MYC siRNA could be titrated in HeLa cells to test whether C-MYC target genes also become desensitised to changes in low C-MYC levels in this system. In HeLa cells, the expression of C-MYC target genes upon double knockdown of CE and C-MYC correlates with C-MYC protein levels, unlike when CE alone is depleted (Figure 4.22 A and B). A possible interpretation of this is that CE has a minor role in regulating C-MYC target genes via regulating C-MYC expression, but in cells with overexpressed C-MYC it has additional influences on C-MYC target genes. In conclusion, C-MYC deregulation sensitises C-MYC target genes to CE depletion, and this is likely to be partially independent of CE regulating C-MYC expression.

4.3 Discussion

4.3.1 Summary

In this chapter it was demonstrated that CE regulates C-MYC expression, potentially via a transcriptional mechanism or regulation of *C-MYC* mRNA stability. CE was shown to regulate C-MYC target genes, and this was partially independent of CE regulating C-MYC expression. Importantly, C-MYC deregulation sensitised C-MYC target genes to CE depletion; target gene expression in normal cells with basal C-MYC levels, or cancer cells with suppressed C-MYC levels, was less affected or unaffected by CE depletion. Since C-MYC is deregulated in a large proportion of cancers, the relationship between CE and C-MYC could be therapeutically relevant.

4.3.2 CE influences RNA pol II on the *C-MYC* gene

CE depletion in HeLa cells resulted in greater RNA pol II occupancy at the pause site on the *C-MYC* gene. A possible interpretation of this result is that CE knockdown causes a defect in pause release, which would support studies showing that CE releases RNA pol II from the pause in vitro (Mandal et al., 2004) and is consistent with the notion that the RNA pol II pausing serves as a 'capping checkpoint'. Indeed, in a previous study, CDK7 inhibition caused a similar change in RNA pol II occupancy on the *C-MYC* gene (Larochelle et al., 2012). Alternatively, since there is also more RNA pol II present at a mid-gene region of *C-MYC* upon CE knockdown, this might indicate a general transcription defect. This could be attributed to CE's ability to induce R-loop formation (Kaneko et al., 2007). Furthermore, lack of the mRNA cap would disrupt recruitment of the cap-binding proteins which recruit splicing factors, and

thus a splicing impairment could cause slower processivity of RNA pol II (increasing RNA pol II occupancy). Consistent with this, knockdown of the CBC caused a decrease in S2p RNA pol II and elevated RNA pol II occupancy across gene bodies (Lenasi et al., 2011) similar to that observed upon CE knockdown in HeLa cells. More studies are required to determine the reasons for, and consequences of, CE-mediated changes in RNA pol II transcription. It would be interesting to determine if CE influences RNA pol II occupancy globally or if there is a preference for certain genes, for example *C-MYC* or C-MYC target genes. Global nuclear run-on followed by sequencing (GRO-seq) could also be performed to analyse how CE knockdown influences *C-MYC* mRNA and global mRNA synthesis. A guanylyltransferase dead mutant of CE (K294A) was shown to retain the ability to alleviate RNA pol II pausing and induce R-loop formation *in vitro* (Kaneko et al., 2007; Mandal et al., 2004), therefore it would be of interest to asses if expressing this mutant could reverse changes in RNA pol II occupancy upon CE depletion. This could help distinguish the effects of CE recruitment and mRNA capping on RNA pol II transcription.

4.3.3 C-MYC target genes are differentially sensitive to CE depletion

In this study, cells carrying deregulated C-MYC were significantly more dependent on CE for C-MYC target gene expression than cells with low C-MYC expression. IMECs which express low endogenous C-MYC levels or HeLa cells in which C-MYC expression was supressed by siRNA were largely unaffected by CE knockdown. This was somewhat surprising given that both endogenous and ectopic C-MYC expression are dependent on CE for their expression. In general, it is intriguing that CE depletion does not have more catastrophic

effects on gene expression. However, as estimated by transcript and protein levels, there is 20% of CE remaining in cells after knockdown. Therefore, in cells with low C-MYC expression, perhaps sufficient amounts of CE remain to maintain transcript capping. Indeed, since studies in this thesis indicate that CE is in excess, the amount of CE remaining after knockdown may suffice for efficient gene expression in these circumstances. To explore this possibility, the cap status of C-MYC target genes could be tested in the presence of CE depletion, for example by immunoprecipitating target gene transcripts using an antibody which specifically recognises the 7-methyl-guanosine cap (Cole and Cowling, 2009a). Notably, *CE* has been described as an essential gene via genome-wide knockout screens using the CRISPR system in seven human cell lines (including HeLa cells), supporting the above hypothesis (Hart et al., 2015; Wang et al., 2014). However, it would be intriguing to attempt CRISPR knockout of *CE* in IMECs to determine whether CE is truly essential for mRNA synthesis and deregulated C-MYC-driven gene expression in this system.

C-MYC overexpression causes a substantial increase in transcriptional load which may render CE availability more limiting. This might be the case for genes expressed above a particular level, or there could be a degree of specificity towards C-MYC target genes, for example if CE is preferentially recruited to C-MYC target genes. In favour of the latter, the effect of C-MYC deregulation on the specific genes analysed is inversely proportional to the effect of CE depletion. Importantly, the global impact of CE knockdown on transcripts in these cells should be investigated by RNA sequencing to determine whether this observation is true on a genome-wide scale. It is also possible that the effect of CE knockdown on C-MYC target genes is via CE

regulating C-MYC expression. However, as shown above, the defect in C-MYC target gene expression in response to CE depletion is greater than that on C-MYC protein expression, demonstrating that CE regulates C-MYC target genes somewhat independently of regulating C-MYC expression. In favour of this, at least in IMECs, CE does not significantly impact global levels of RNA pol II phosphorylation unlike C-MYC, consistent with CE impacting C-MYC target genes in other ways. In light of findings in this thesis, this could be due to CE being a co-factor in C-MYC-driven gene expression. However, the possibility remains that secondary effects of CE knockdown alter the expression of C-MYC target genes. To determine if CE is truly a transcriptional co-factor of C-MYC, *CE* could be fused to the *C-MYC* DBD (which alone is insufficient for C-MYC-driven transcription) via a flexible linker region and its ability to transactivate C-MYC target genes tested. This would determine whether localising CE in proximity of C-MYC target genes and paused RNA pol II was sufficient to induce partial activation of gene expression.

It is worth noting that during the preparation of this thesis, the mechanism of C-MYC-mediated mRNA capping was investigated in an independent study, with a focus on specific C-MYC target genes involved in WNT signalling (Posternak et al., 2017). RNMT depletion but not CE depletion significantly reduced capping of these transcripts (as determined by N7-methylguanosine cap precipitation) relative to total mRNA levels. From this the authors concluded that RNMT but not CE was rate-limiting in C-MYC-driven mRNA capping. However, mRNA levels were not reported; only capped mRNA relative to total mRNA levels. Therefore any alterations resulting from transcriptional or mRNA stability changes upon CE depletion were masked, and

the relative contribution of CE and RNMT to net C-MYC target gene expression is still not known.

Regardless of whether CE directly or indirectly regulates C-MYC target genes, the differential sensitivity of target genes depending on C-MYC levels means these observations are exciting from a therapeutic point of view, since an ideal chemotherapy for C-MYC-driven cancers should impact cancer cells with deregulated C-MYC but not healthy cells with basal C-MYC activity. Therefore, in the following chapter the effect of CE inhibition on cell transformation will be tested to determine whether CE should be considered as a therapeutic target.

Chapter 5 : Deregulated C-MYC induces mRNA capping enzyme dependency

5.1 Introduction

C-MYC overexpression or hyperactivation increases cell proliferation and invasiveness, and contributes to cell transformation. Deregulation of C-MYC occurs in many cancer types and in over 50% of all cancer cases. Despite extensive efforts to target C-MYC via a plethora of avenues, there are still no therapies available which do so. This is partially because C-MYC, being a transcription factor, has no active site which can be readily inhibited by small-molecule compounds. Therefore, interest has been maintained in targeting enzymatic co-factors of C-MYC. Moreover, compensatory mechanisms upon inhibiting C-MYC have been reported (Donato et al., 2016; Fong et al., 2015; Lu et al., 2015; Rathert et al., 2015), suggesting that combination therapies may be required to treat C-MYC-dependent cancers.

In previous chapters it was shown that C-MYC promoted CE recruitment to transcription complexes. Moreover, C-MYC target genes were highly sensitive to CE depletion in the presence of supraphysiological C-MYC, but less so in the presence of basal C-MYC levels, indicating that cells with overexpressed C-MYC are particularly sensitive to CE inhibition. CE was also shown to regulate the expression of C-MYC itself. Therefore, inhibiting CE has the potential to diminish both C-MYC expression and C-MYC function. In this chapter it will be investigated whether CE is required for transformative

properties conferred by overexpressed C-MYC to determine if CE should be considered as a therapeutic target in C-MYC-driven cancers.

5.2 Results

5.2.1 CE knockdown specifically reduces IMEC/C-MYC cell number

As previously mentioned, C-MYC increases cell proliferation. CE is required for C-MYC target gene expression in cells with overexpressed C-MYC but not in those retaining normal control of C-MYC. Therefore, it was tested if CE inhibition affected IMEC/vec and IMEC/C-MYC cell number over time, which is an indication of their proliferative potential. Cells were transfected with CE siRNA and counted after 72 hours (Figure 5.1 A). Consistent with previous studies, overexpression of *C-MYC* in IMECs increases cell number (Cowling et al., 2007), indicating that C-MYC increased proliferation in these cells. There was a 20% reduction in IMEC/C-MYC cell number in response to CE inhibition, but IMEC/vec cell number was unaffected. This suggests that CE is required for C-MYC-induced proliferation, but not basal IMEC proliferation, and demonstrates that C-MYC overexpression sensitises IMECs to CE depletion. In order to truly determine if proliferation is affected, cell doubling time should be measured. Note that although there is some variation in IMEC/C-MYC cell number after three days, the trend upon CE knockdown remains much the same between biological replicates (Figure 5.1 B). The variation may be due to cell passage number or a counting error when first seeding cells.

The standard IMEC culture medium is serum-free and is instead supplemented with specific growth factors (Table 2.2). However anchorage-independent growth assays (see below) require further supplementation with 5% fetal bovine serum (FBS). C-MYC increases IMEC cell number to a similar extent in the presence or absence of FBS (Figure 5.1 C), indicating that C-MYC

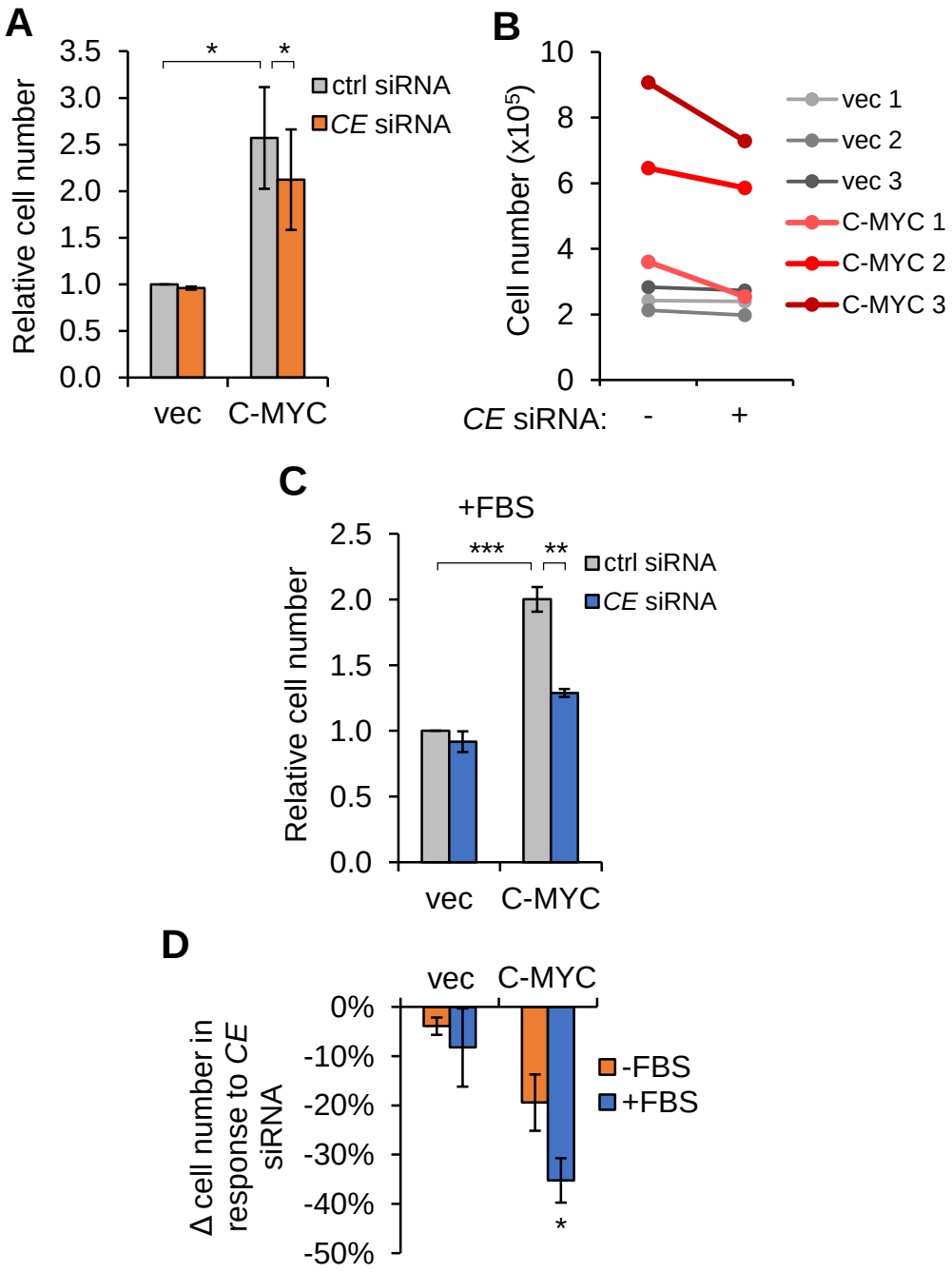

Figure 5.1: CE depletion affects IMEC/C-MYC cell number. (A) 1.1x10^5 IMEC/vec or IMEC/C-MYC were transfected with *CE* siRNA or a non-targeting control (ctrl) siRNA and counted after 72 hours. Error bars represent standard error of the mean, n=3. (B) As in (A) except cell number after 72 hours is depicted for three biological replicates (1-3). (C) as in (A) except cells were maintained in media containing FBS. Error bars represent standard error of the mean, n=3. (D) Change (Δ) in IMEC/vec and IMEC/C-MYC cell number following CE depletion (relative to ctrl siRNA) in the presence or absence of FBS. Error bars represent standard error of the mean, n=3. Significance calculated by Student's t-test, *p≤0.05; **p≤0.01; ***≤0.001.

still induces IMEC proliferation in these culture conditions. The cell number of IMEC/vec maintained in FBS was not significantly affected by CE depletion, whereas IMEC/C-MYC cell number was reduced by 40%. This suggests that CE is required for C-MYC-induced proliferation, even more so when cells are cultured in FBS (Figure 5.1 D). C-MYC expression was not noticeably altered by culturing cells in FBS (Figure 5.2 B) although FBS noticeably increased IMEC proliferation (data not shown). It is likely that FBS stimulates growth factor signalling and IMEC proliferation, which could be coupled with increased global gene expression, and this may increase cellular dependency on CE.

5.2.2 CE depletion upregulates CDK inhibitors

A possible explanation for the above observations is that CE depletion induces apoptosis in IMEC/C-MYC. However, there were no obvious morphological changes that would indicate this (Figure 5.2 A) and no floating cells were observed in petri dishes. Consistent with this, poly ADP ribose polymerase (PARP) cleavage, a common indicator of apoptosis, was not detected in cells cultured with or without FBS (Figure 5.2 B). Alternatively, cell cycle progression could be inhibited by CE depletion. The CDK inhibitors p21 and p27 negatively regulate the G1-S transition in the cell cycle and are repressed by C-MYC (Gartel et al., 2001a; Seoane et al., 2002; Yang et al., 2001). Therefore it was tested whether CE depletion altered p21 and p27 expression. Although only p21 was reproducibly suppressed by C-MYC in IMECs, CE depletion in IMEC/C-MYC upregulated both p21 and p27 expression, whereas that in IMEC/vec was unaffected (Figure 5.3 A and B). Accordingly, C-MYC does not always impact p27 expression (Vlach et al., 1996). Regardless, this indicates that CE knockdown restrains C-MYC-driven

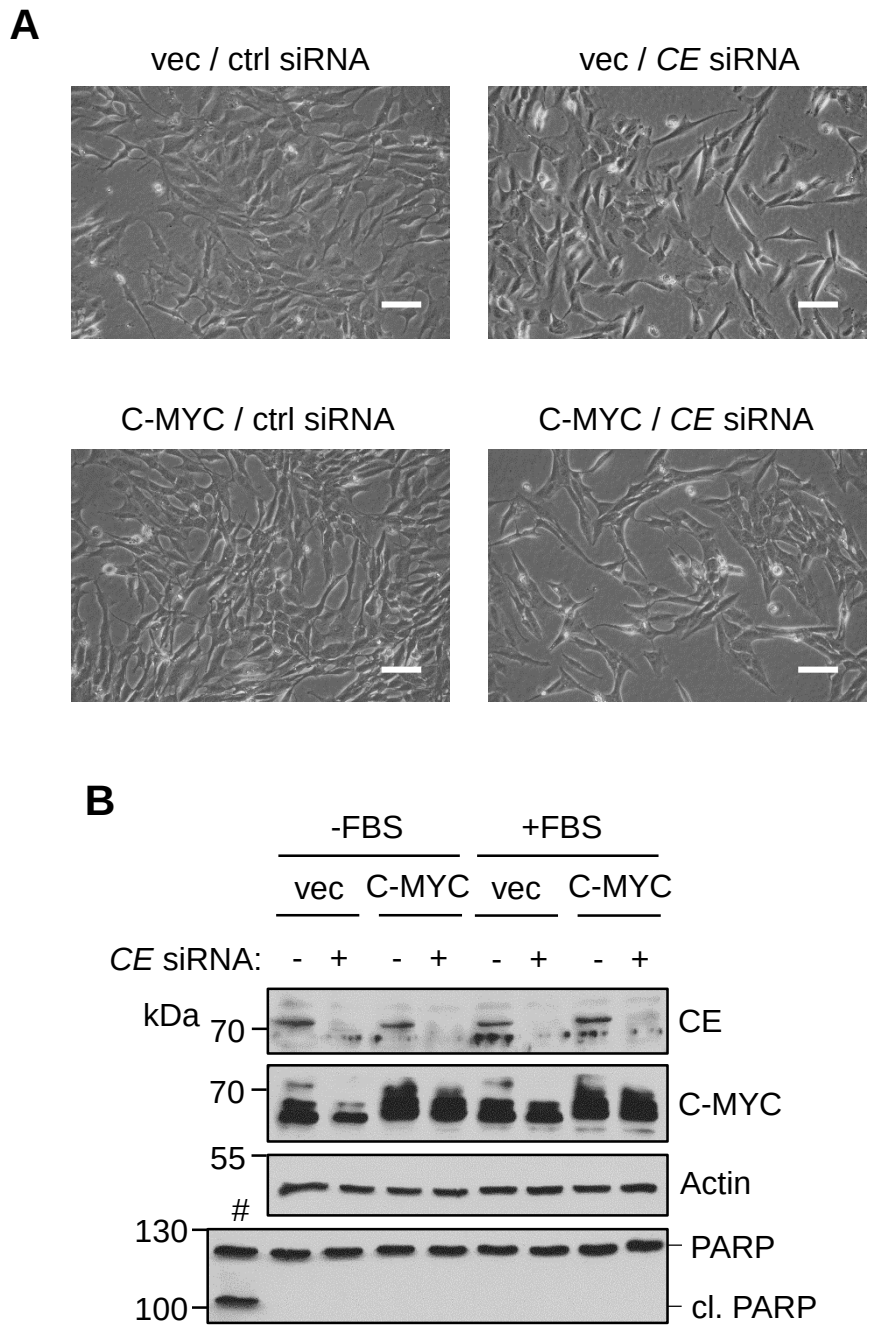

Figure 5.2: Apoptosis is not detected upon CE depletion.
(A) Bright-field images of IMEC/vec and IMEC/C-MYC (maintained in media supplemented with FBS) transfected with *CE* siRNA or a non-targeting control siRNA (ctrl). Micrographs were taken after 72 hours. Scale bar represents 100µm. (B) IMEC/vec and IMEC/C-MYC maintained with or without FBS were transfected with *CE* siRNA or a non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting. # IMEC/C-MYC treated with 10µM MG132 for 24 hours as a positive control for apoptosis. Representative of two independent experiments.

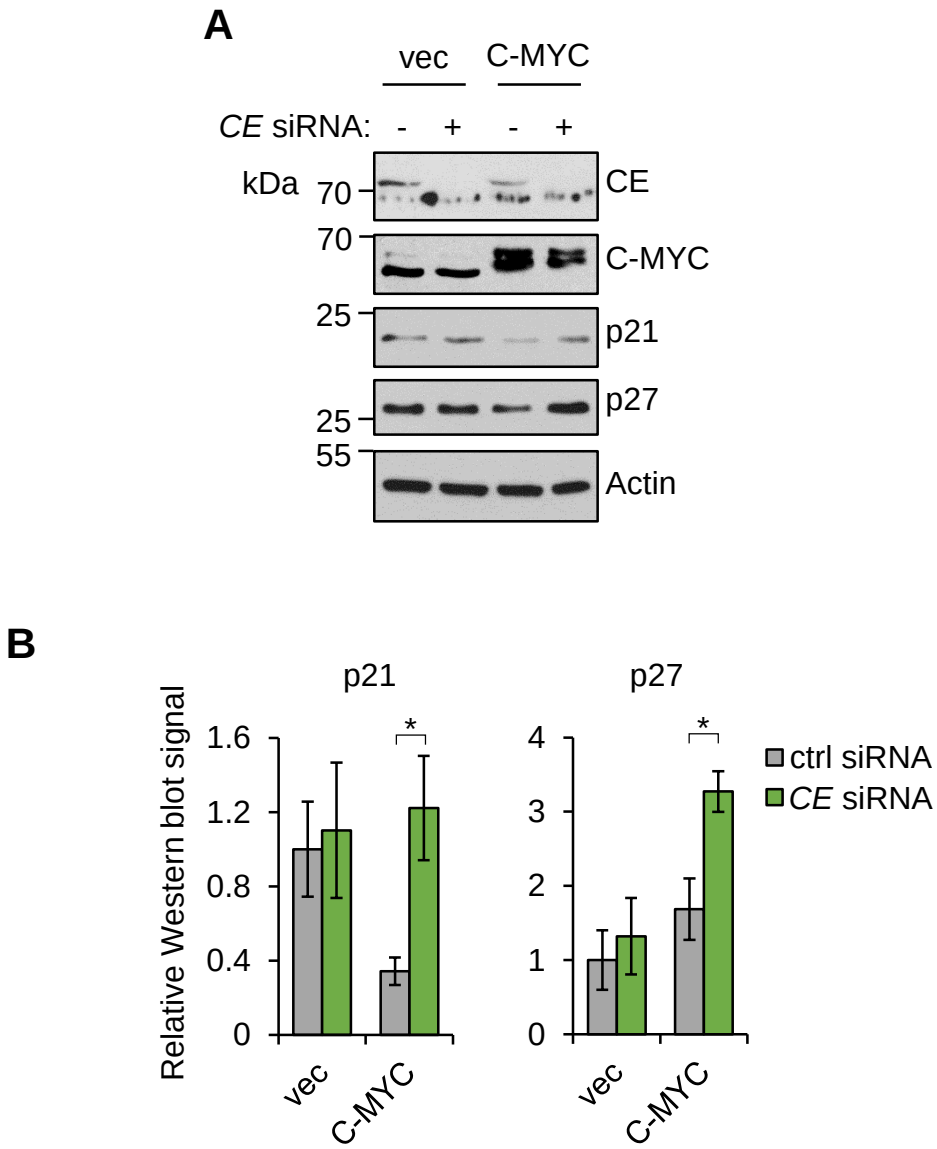

Figure 5.3: CE depletion induces upregulation of CDK inhibitors in the presence of C-MYC overexpression. (A) IMEC/vec and IMEC/C-MYC were transfected with *CE* siRNA or a non-targeting control siRNA (-). Protein was extracted from cells after 72 hours and analysed by Western blotting. (B) Densitometry was performed using ImageJ software to quantify p21 and p27 Western blot signal (normalised to actin) following transfection of siRNA as above. Similar results were obtained from IMECs cultured with and without FBS, therefore results from cells cultured in both conditions are pooled in this panel. Error bars represent standard error of the mean, n≥4. Significance calculated by Student's t-test, *p≤0.05.

cell cycle transit in IMECs. To confirm that CE depletion causes a cell cycle defect, DNA content and cell cycle phase should be analysed following CE knockdown by fluorescence-activated cell sorting (FACS).

5.2.3 CE is required for C-MYC-induced transformation

In IMECs, overexpressed C-MYC induces anchorage-independent cell growth (Cowling et al., 2007). Non-transformed cells undergo anoikis (cell death resulting from extracellular matrix detachment) when not adhered to a solid surface, which is a strategy to eliminate misplaced cells and prevent metastasis. Conversely, transformed cells are often able to evade anoikis and proliferate without attachment, which can cause metastases in vivo. Therefore, the ability of cells to undergo anchorage-independent cell growth in vitro is indicative of their aggressiveness and metastatic potential in vivo (Mori et al., 2009). It was thus investigated whether CE inhibition affected C-MYC-driven anchorage-independent growth. As alluded to above, IMECs maintained in FBS-supplemented medium were used for this assay. IMEC/vec and IMEC/C-MYC were transfected with CE siRNA prior to being plated in media containing agar. This sets semi-solid and thus suspends cells in medium, allowing the ability of cells to form anchorage-independent colonies to be investigated. Similar to previous studies (Cowling and Cole, 2007a; Cowling et al., 2007), 50% of IMEC/C-MYC formed anchorage-independent colonies after 9-15 days (Figure 5.4 A). CE depletion reduced the number of IMEC/C-MYC anchorage-independent colonies by 30% (Figure 5.4 A), and the number of larger colonies over 50μm in diameter by 60% (Figure 5.4 B). Note that the variation in data for larger colonies lies mainly in the incidence of colony formation rather than variation in the effect of CE depletion (Figure 5.4 C), which could be due to

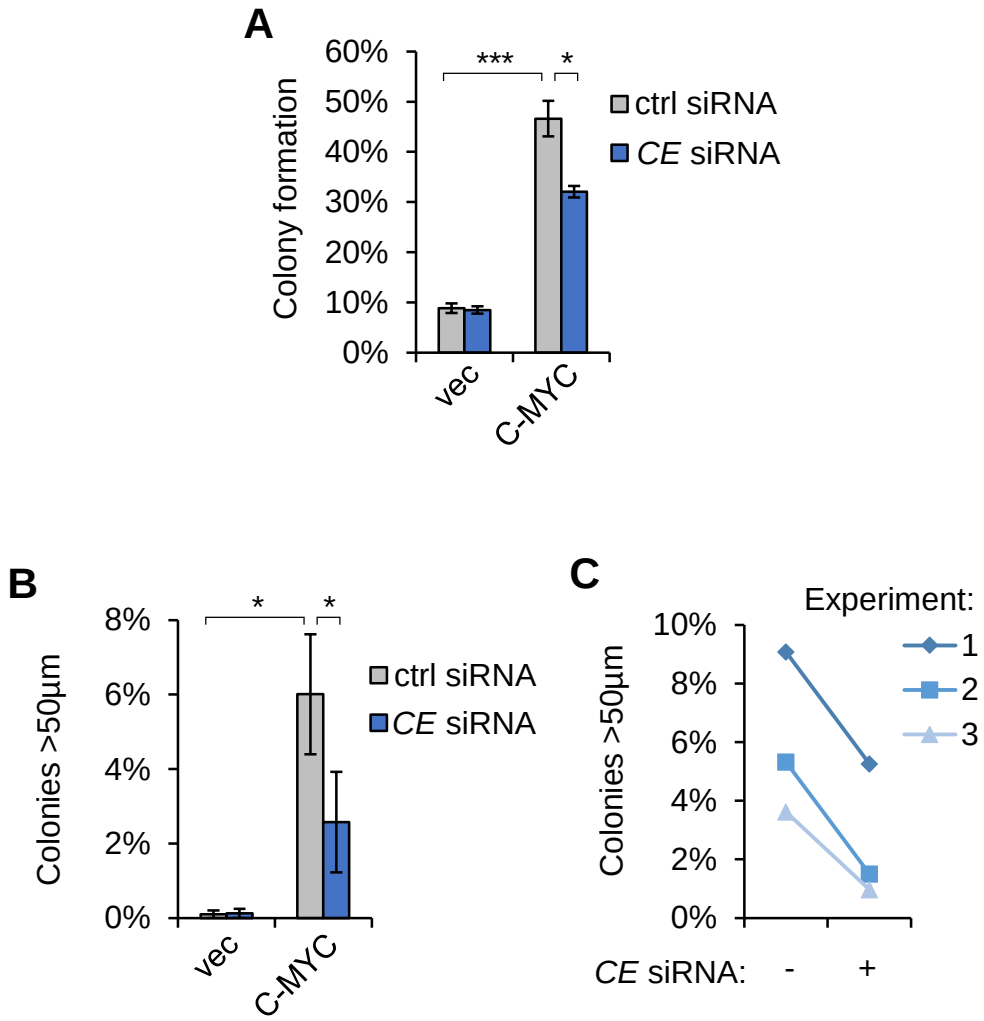

Figure 5.4: CE is required for C-MYC-driven transformation in IMECs. (A) IMEC/vec and IMEC/C-MYC maintained with 5% FBS were transfected with *CE* siRNA or a non-targeting control (ctrl). After 72 hours cells were seeded in suspension. Colonies were scored using a graticule after 9-15 days. The percentage of cells that had formed colonies >20µm are reported. Error bars represent standard error of the mean, n=3. (B) As in (A) except the percentage of cells that formed colonies >50µm are reported. Error bars represent standard error of the mean, n=3. (C) as in (B) except each IMEC/C-MYC biological replicate shown. Significance calculated by Student's t-test, ***p≤0.001; *p≤0.05. Figure continued overleaf.

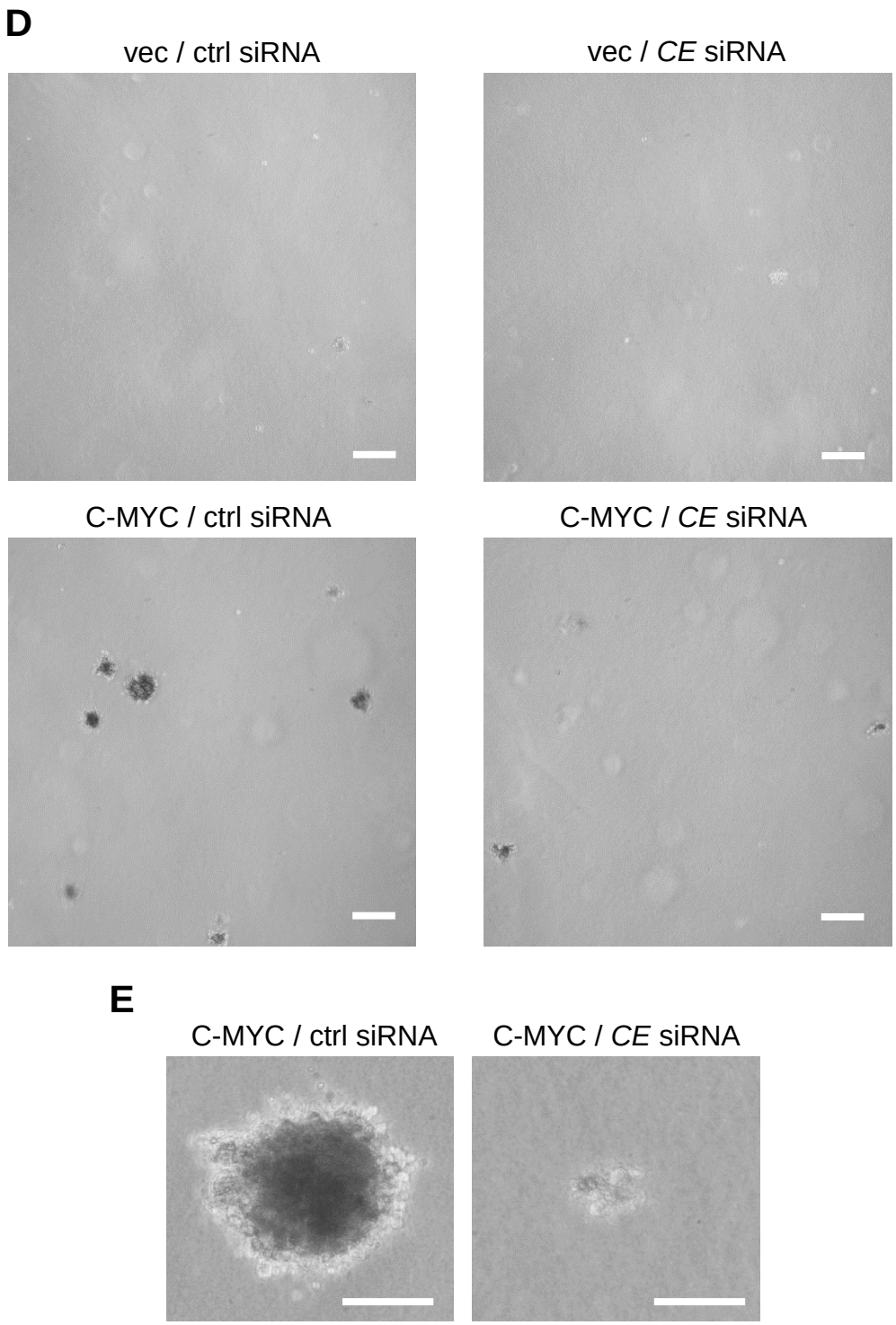

Figure 5.4 continued: IMECs with deregulated C-MYC require CE for cell transformation. (D) Bright-field images of IMEC/vec and IMEC/C-MYC transfected with *CE* siRNA or non-targeting control (ctrl) and suspended in soft agar. Micrographs were taken one month after cells were seeded in suspension. Scale bar represents 200µm. (E) as in (D) except scale bar represents 100µm.

colonies being counted different lengths of time after plating, cell passage number or slight difference in medium agar density. Representative micrographs were taken after one month (Figure 5.4 D and E). In summary, CE is important for C-MYC-driven anchorage-independent cell growth and thus C-MYC-dependent cell transformation.

5.2.4 High *CE* expression predicts poor breast cancer patient outcome

Since CE expression influenced C-MYC-induced transformation in IMECs, it was investigated if *CE* transcript levels correlated with breast cancer patient prognosis. The online 'Kaplan Meier' plotter was used which utilises microarray from patient tumour samples together with patient survival information (Gyorffy et al., 2010). For high/low gene expression cut-off selection, the programme tested each percentile of expression between the lower and upper quartiles, and the best performing threshold was selected (indicated on figures). Relapse-free survival (the time after successful treatment until relapse) and post-progression survival (survival time after a tumour spreads or worsens) were analysed (Figure 5.5 A). Expression of topoisomerase 2A (*TOP2A*), an established breast cancer prognostic marker, was investigated using the same search parameters as a positive control. There was little correlation between *C-MYC* expression and patient outcome. This is not necessarily surprising considering that *C-MYC* does not have to be transcriptionally upregulated to be oncogenic; changes in C-MYC protein levels and activity can be sufficient (Murphy et al., 2008; Wang et al., 2011). *CE* expression in breast tumours was inversely correlated with relapse-free survival (most so in those with the basal-like tumour subtype) and with post-progression survival (Figure 5.5 A). *CE* levels were analysed by the same means in ovarian,

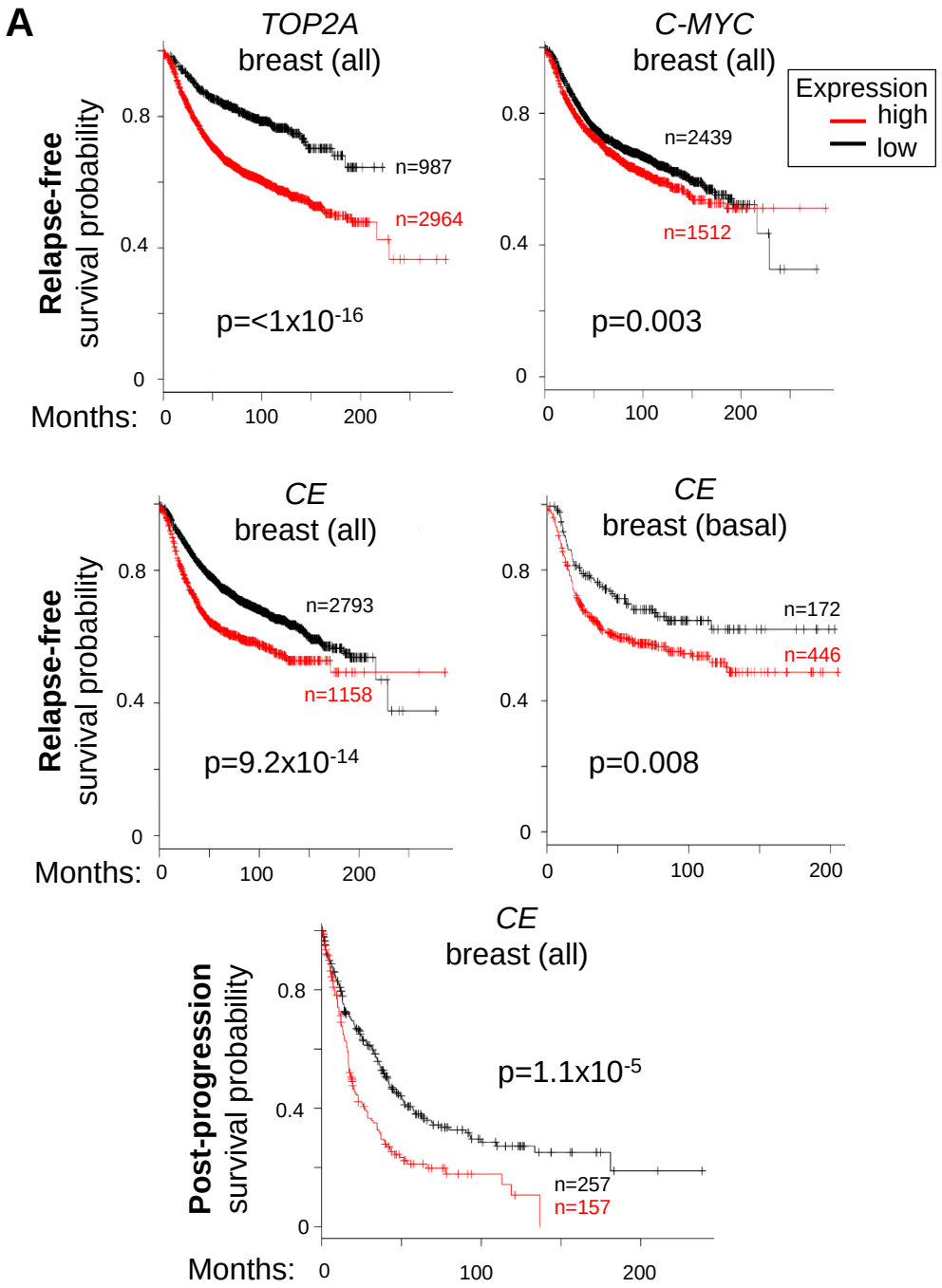

Figure 5.5: *CE* expression correlates with poor breast cancer patient outcome. (A) The online survival analysis tool 'Kaplan Meier Plotter' (www.kmplot.com) was used to determine how high (red) and low (black) expression of *TOP2A, C-MYC* and *CE* mRNA in tumours correlated with relapse-free or post-progression survival of patients with breast cancer in general or those with the basal sub-type of breast cancer. Sample sizes and cut-off values are indicated on graphs. Error bars represent the hazard ratio (95% confidence interval). Significance (p) was calculated using the log-rank test. Figure continued overleaf.

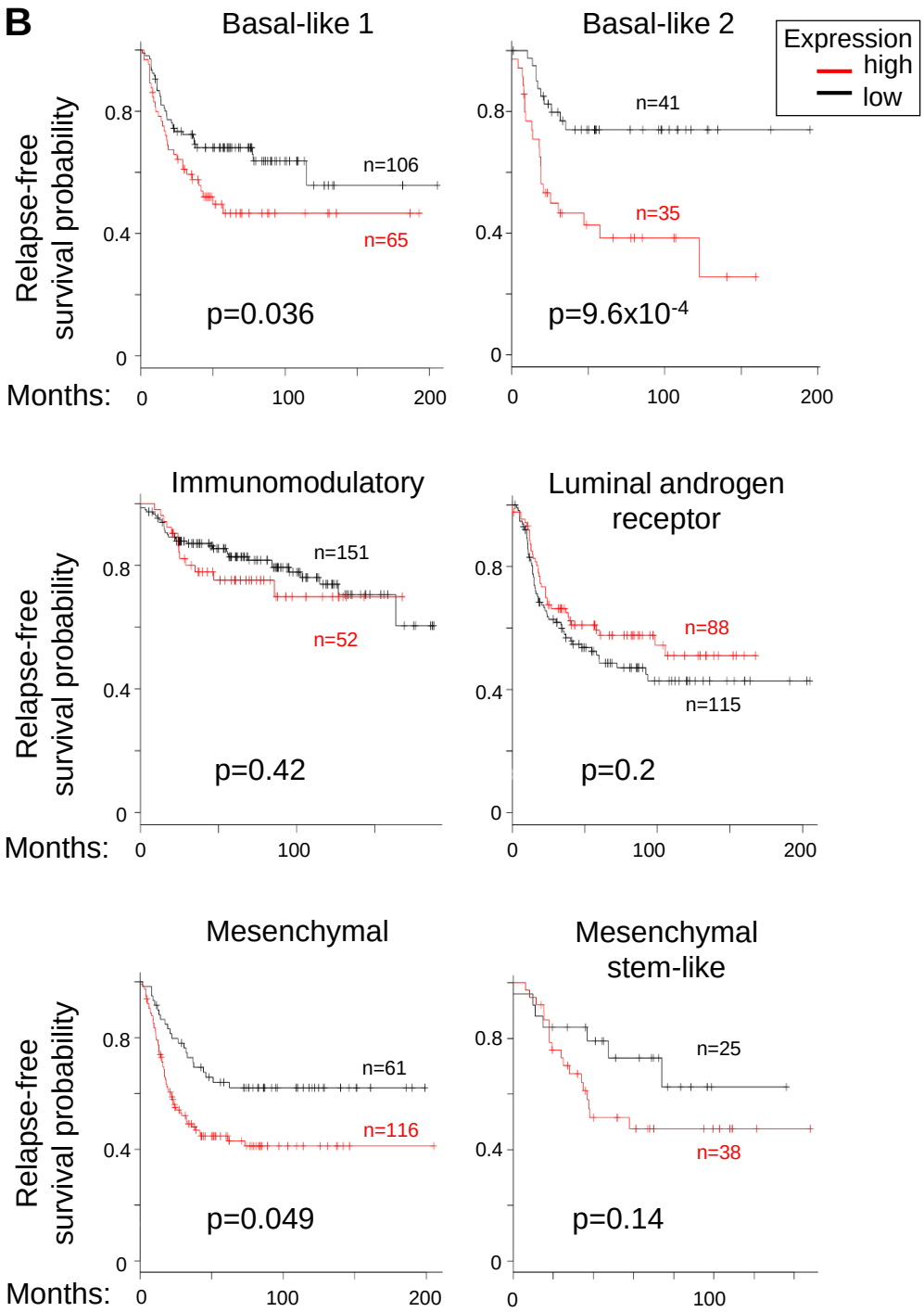

Figure 5.5 continued: *CE* expression correlates with poor breast cancer patient outcome. (B) as in (A) except high and low *CE* mRNA levels in tumours is plotted against the relapse-free survival of patients with specific subtypes of triple-negative breast cancer. Sample sizes and cut-off values are indicated on graphs. Error bars represent the hazard ratio (95% confidence interval). Significance (p) was calculated using the log-rank test.

C

TNBC subtype	Enriched GO terms/canonical pathways
BL1	Cell cycle, DNA replication interactome, G2 pathway, RNA polymerase, ATR/BRCA pathway, G1 to S cell cycle.
BL2	EGF pathway, NGF pathway, MET pathway, WNT β-catenin pathway, IGF1R pathway, Glycolysis/gluconeogenesis.
M	IGF/mTOR pathway, ECM pathway, Regulation of actin by RHO, WNT pathway, ALK pathway, TGFβ pathway.

Figure 5.5 continued: *CE* expression correlates with poor breast cancer patient outcome. (C) Enriched GO terms for specific TNBC subtypes. Only the subtypes in which high *CE* expression predicts a poor outcome in are listed. Data from Lehmann et al. (2011). BL1, basal-like 1; BL2, basal-like 2; M, mesenchymal. See list of abbreviations for other definitions.

D

C-MYC co-factors | C-MYC induced genes | C-MYC repressed genes

Type	*C-MYC*	*CE*	*CDK7*	*TIP49*	*CDK9*	*NME1*	*FBL*	*NCL*	*ODC*	*CCND1*	*p27*	*p21*	*ACTB*	*TOP2A*
All	0.003	0.000	0.000	0.000	0.100	0.000	0.001	0.000	0.140	-0.013	0.000	-0.031	0.000	0.000
Basal	0.260	0.008	0.000	0.000	0.016	0.002	0.003	0.210	0.000	0.070	0.360	-0.002	0.000	0.310
BL1	0.049	0.036	0.000	0.007	0.032	0.001	0.067	-0.017	0.380	0.010	0.048	-0.058	-0.097	0.068
BL2	-0.059	0.001	0.001	0.002	-0.018	0.058	0.098	0.032	-0.014	-0.074	0.360	0.120	0.000	-0.094
M	-0.046	0.005	0.001	0.002	0.310	0.087	0.004	0.430	-0.001	0.210	0.260	0.300	-0.007	0.110
All (PPS)	0.170	0.000	0.260	0.200	0.250	-0.029	-0.026	0.003	0.012	0.320	0.140	0.320	-0.027	0.002

Colour scale	Significance (p/transformed p)	Prognosis (high expression)
	0-0.0001	Unfavourable
	0.0001-0.05	Unfavourable
	0.05-0.1	Unfavourable (tentative)
	0.1-1	No significant trend
	(-0.05)-(-0.1)	Favourable (tentative)
	0-(-0.05)	Favourable

Figure 5.5 continued: *CE* expression correlates with poor breast cancer patient outcome. (D) as in (A) and (B) except analysis extended to include C-MYC co-factors and target genes. Figure depicts how high expression of these genes in tumours predicts breast cancer patient relapse-free survival or post-progression survival (PPS). A correlation between high expression of a gene and poor prognosis is illustrated by a p value of 0-0.1 (red/pink). A correlation between high expression of a gene and favourable prognosis is illustrated by a transformed p value of 0-(-0.1) (green). Relapse-free survival data is included for breast cancer patients (all), those with basal-like breast cancers, and those with specific subtypes of TNBC. Note that the C-MYC co-factors listed are also transcriptionally regulated by C-MYC, and *CCND1* can be induced or repressed by C-MYC in a system-dependent manner. *ACTB* (beta-actin) was included in analyses as a randomly selected gene. Significance (p) was calculated using the log-rank test. BL1, basal-like 1; BL2, basal-like 2; M, mesenchymal.

lung and gastric cancers, but a correlation with survival was absent or less apparent (data not shown). The basal-like breast cancer tumour subtype (characterised by high expression of markers such as cytokeratins 5, 6 and 17) comprises 15-20% of all breast cancers and is biologically and clinically aggressive (Alluri and Newman, 2014). 80% of basal-like breast cancers are also classed as 'triple-negative', meaning they lack expression of oestrogen receptor (*ER*), progesterone receptor (*PR*) and human epidermal growth factor receptor 2 (*HER2*) thus rendering them resistant to available targeted therapies (Bertucci et al., 2012). Further analysis was conducted to analyse how *CE* expression correlated with survival in patients with different subtypes of triple-negative breast cancer (TNBC) (ontology defined by gene expression profiling) (Lehmann et al., 2011). The 'basal-like 1', 'basal-like 2', and 'immunomodulatory' TNBC subtypes are classified as basal-like. Intriguingly, *CE* expression was particularly anti-correlative with survival in patients with basal-like 1/2 and mesenchymal subtypes (Figure 5.5 B-C). Of interest, basal-like 1 tumours exhibit overexpression of proliferation-associated genes including *C-MYC* (Lehmann et al., 2011). Furthermore, basal-like 2 tumours display gene expression signatures associated with growth factor signalling, and (as previously discussed) C-MYC is commonly activated by such signalling pathways. Mesenchymal tumour subtypes are enriched for gene expression signatures related to mTOR (mammalian target of rapamycin) which activates cap-dependent translation. This tumour subtype is also characterised by epithelial to mesenchymal transition (EMT) markers. EMT is associated with tumour invasiveness and metastasis, and is promoted by C-MYC in mammary epithelial cells (Cho et al., 2010; Cowling and Cole, 2007a). Consistent with C-MYC activity having a role in these tumour subtypes, high expression of C-MYC

co-factors including *CDK7* and *TIP49*, and C-MYC induced genes including *NME1* and *FBL,* also correlated with poor patient prognosis (Figure 5.5D). Notably, the trend between patient survival and C-MYC co-factor expression was highly similar to that observed with *CE* expression (Figure 5.5D). A panel of breast cancer cell lines with defined molecular ontologies could be tested to determine whether these particular subtypes are responsive to CE inhibition. Moreover, it would be of interest to determine if *CE* expression correlates with survival in patients with high-*C-MYC* expressing tumours, and in cancers known to driven by *MYC* family members such as Burkitt's lymphoma and neuroblastoma.

Of note, breast cancers in general to not exhibit elevated *CE* expression (Shaul et al., 2016); perhaps breast cancer cells that inadvertently have elevated *CE* have a survival advantage. On the other hand, some B cell lymphoma cancer cell lines (including Burkitt's lymphomas) which are known to be driven by C-MYC (Bahram et al., 2000; Bhatia et al., 1993; Cai et al., 2015; Li et al., 2003b; Nguyen et al., 2017), and lymphoma primary tumours, have exhibited heightened *CE* expression (Shaul et al., 2016). This suggests that elevated CE activity may be important for C-MYC's oncogenicity in B cells, and this warrants further investigation.

5.2.5 Examining cellular effects of CE knockdown in HeLa cells

In the previous chapter it was shown that C-MYC target genes were sensitive to CE depletion in HeLa cells with overexpressed C-MYC, but suppression of C-MYC desensitised target genes to CE inhibition. To determine if changes in C-MYC target gene expression reflected changes in cell number

(as in IMECs), HeLa cells were transfected with combinations of *C-MYC* siRNA and *CE* siRNA as before and cells were counted after 72 hours. Unexpectedly, C-MYC knockdown, CE knockdown and C-MYC/CE double knockdown had no effect on HeLa cell number (Figure 5.6 A) suggesting that this did not impact cell proliferation under these conditions. This is contradictory to prior studies which showed that *C-MYC* siRNA reduced proliferation of HeLa cells after 72 hours (Cappellen et al., 2007) and *CE* siRNA induced HeLa cell apoptosis after 48 hours (Chu and Shatkin, 2008). Potential causes of this discrepancy include the use of different cell passages or different knockdown efficiencies. Perhaps C-MYC-dependent cells are particularly dependent on CE, and those not dependent on C-MYC are less so. Alternatively, C-MYC may promote HeLa cell proliferation without causing net changes in cell number, since overexpressed C-MYC in some systems has paradoxical roles in promoting both cell growth and apoptosis (Murphy et al., 2008; Tansey, 2014). In favour of this, cleaved PARP was detected in control transfected cells more so than in other conditions (Figure 5.6 B). Additionally, less floating cells were observed in petri dishes after transfection of *CE* siRNA or *C-MYC* siRNA compared to control transfected cells (data not shown), suggesting that HeLa cells may be more primed for apoptosis in the presence of high C-MYC or CE levels. To test this hypothesis, additional markers of apoptosis should be analysed. For example, Annexin V staining could be performed to analyse cells undergoing apoptosis, and the nucleic acid stain SYTOX green could be used which only permeates dead cells.

Interestingly, the colour of culture medium was noticeably less yellow/orange upon *CE* and *C-MYC* knockdown compared to control cells (data

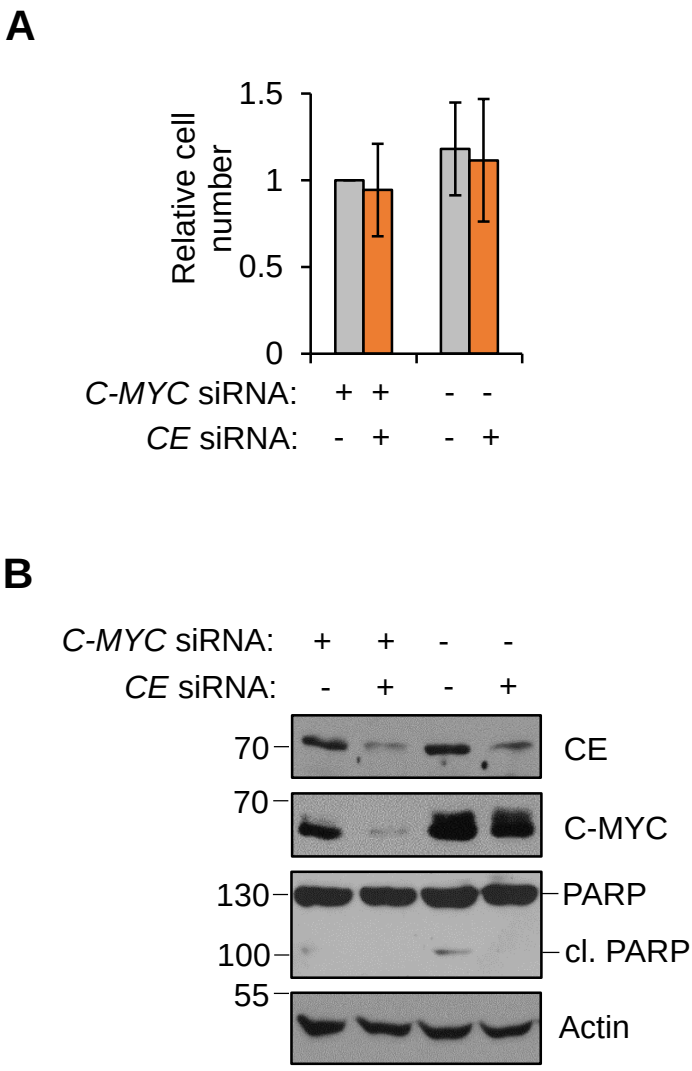

Figure 5.6: Investigating how CE and C-MYC depletion affects HeLa cells. (A) HeLa cells were transfected with combinations of *CE* siRNA, *C-MYC* siRNA and non-targeting control siRNA (-) before being counted after 72 hours. Error bars represent standard error of the mean, n=5. (B) Cells were transfected with siRNA as in (A) and analysed by Western blotting. Representative of three independent experiments. Figure continued overleaf.

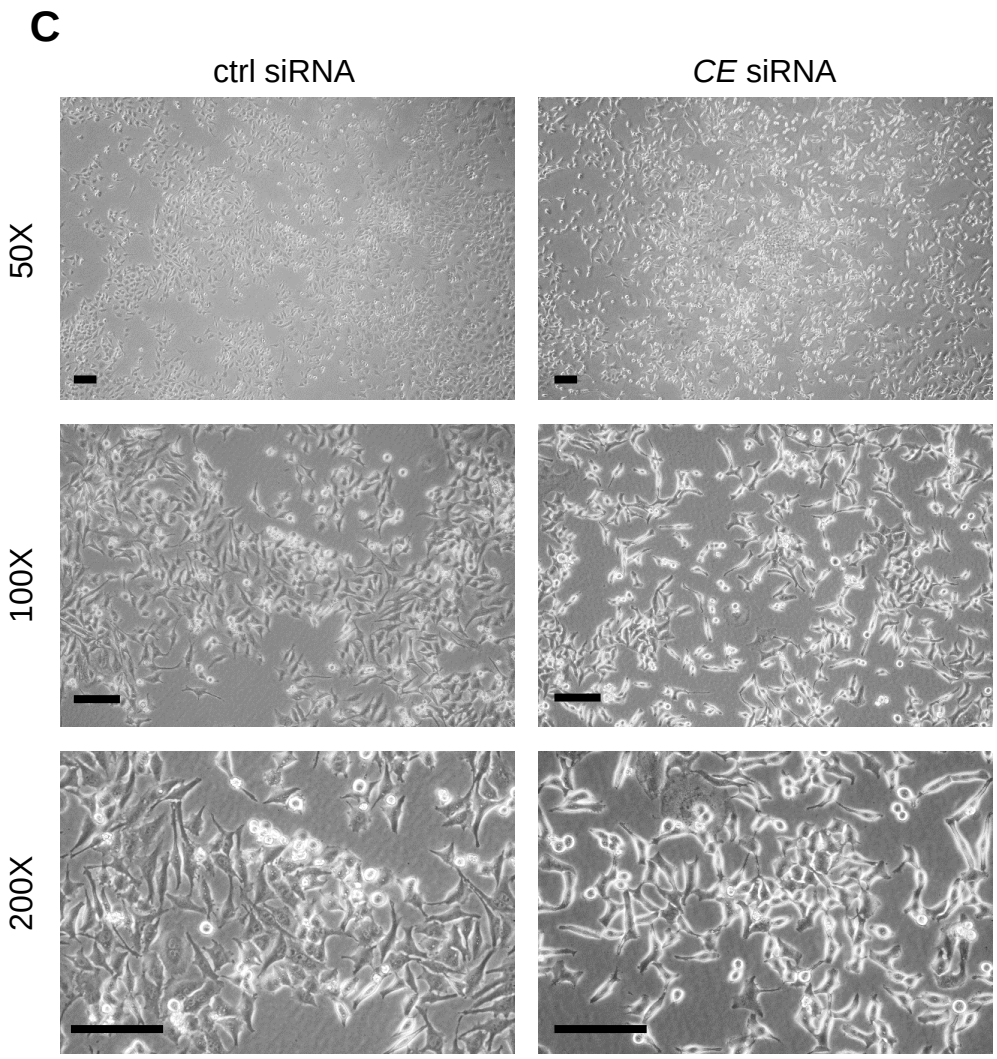

Figure 5.6 continued: Investigating how CE and C-MYC depletion affects HeLa cells. (C) Bright-field images of HeLa cells transfected with *CE* siRNA or a non-targeting control siRNA (ctrl), taken at the indicated magnifications. Micrographs were taken 72 hours post-siRNA transfection. Scale bar represents 100µm. Figure continued overleaf.

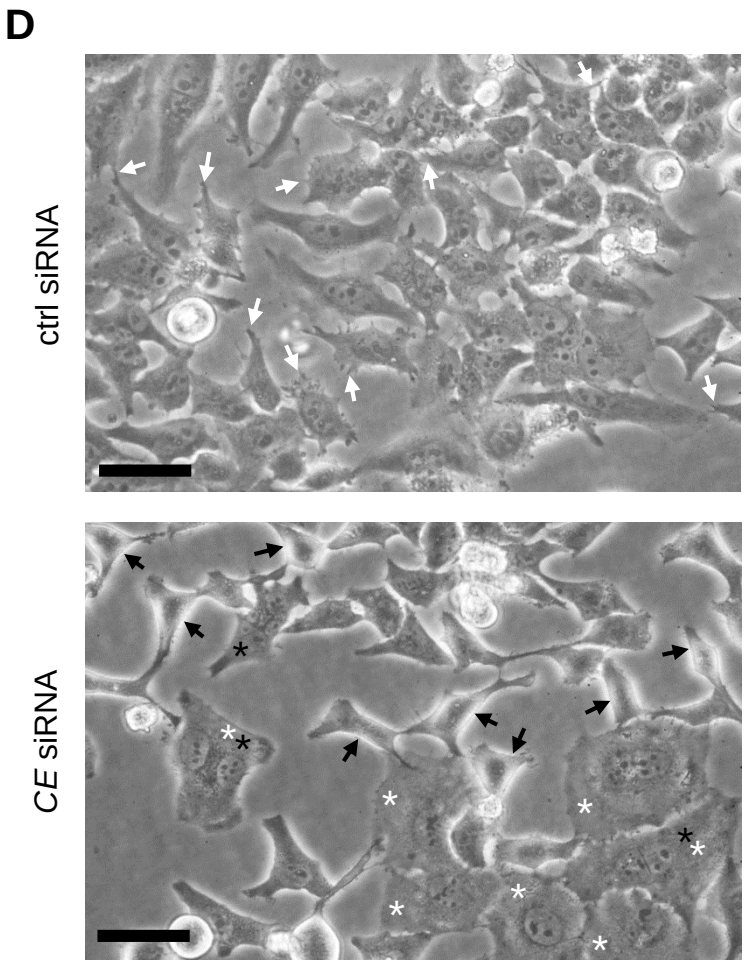

Figure 5.6 continued: Investigating how CE and C-MYC depletion affects HeLa cells. (D) As in (C) except taken at 400X magnification. White arrow=cell protrusion; white asterix=large, flat cell; black arrow=refractile cell; black asterix=multi-nucleic cell. Scale bar represents 50µm. Figure continued overleaf.

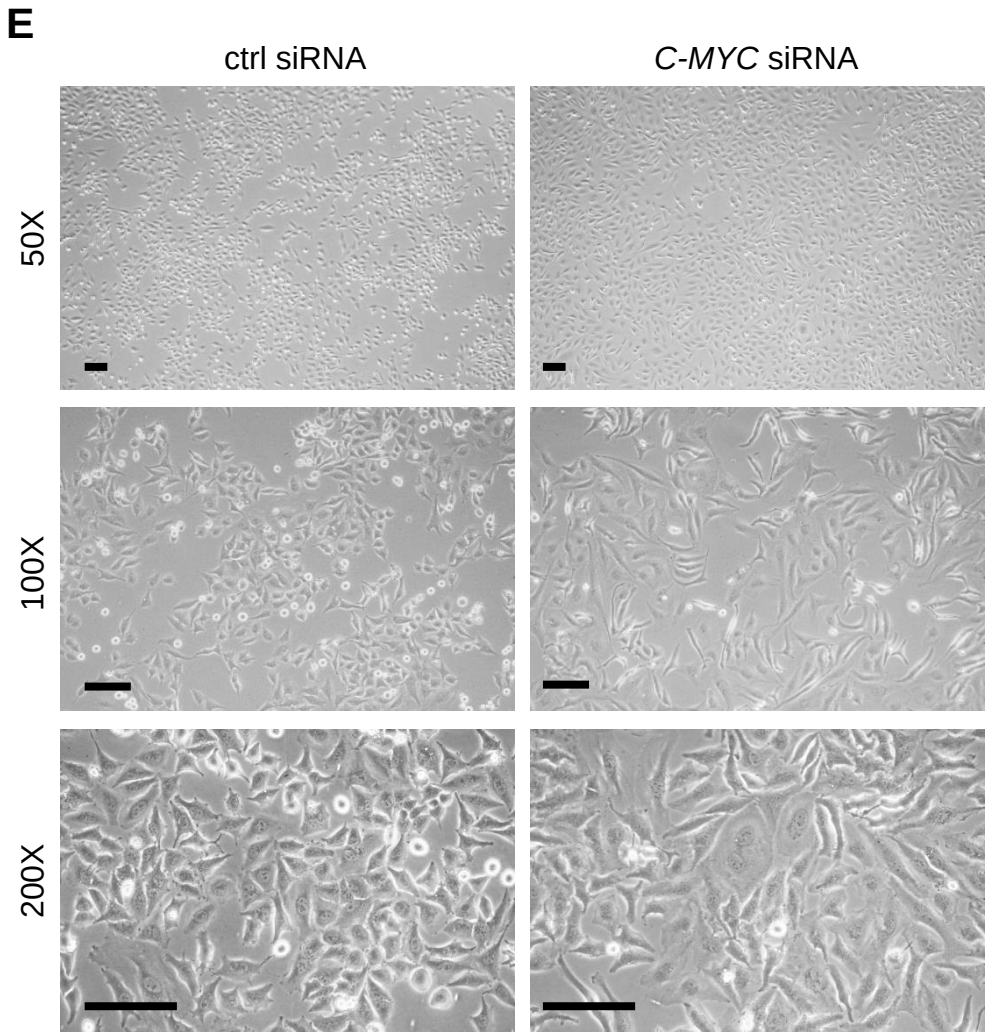

Figure 5.6 continued: Investigating how CE and C-MYC depletion affects HeLa cells. (E) Bright-field images of HeLa cells transfected with *C-MYC* siRNA or a non-targeting control siRNA (ctrl) taken at the indicated magnifications. Micrographs were taken 60 hours post-siRNA transfection. Scale bar represents 100µm. Figure continued overleaf.

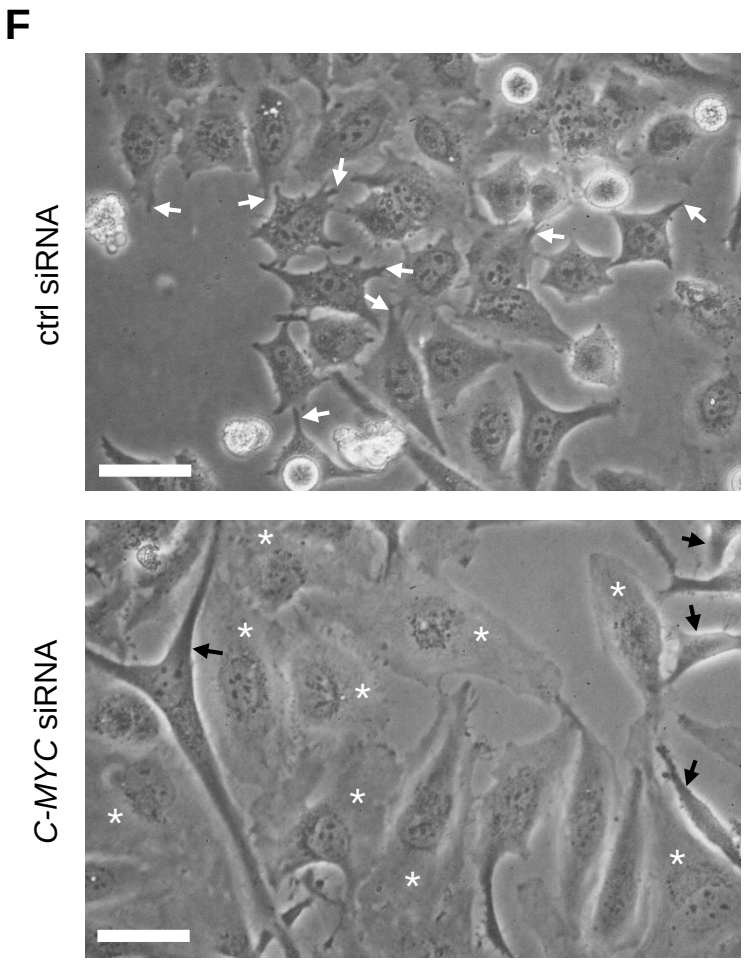

Figure 5.6 continued: Investigating how CE and C-MYC depletion affects HeLa cells. (F) As in (E) except taken at 400X magnification. White arrow=cell protrusion; white asterix=large, flat cell; black arrow=refractile cell. Scale bar represents 50µm.

not shown) despite no alteration in cell number, indicating that control cells were more metabolically active. C-MYC is known to alter cell metabolism in many ways, including enhancing cell glycolysis which helps cancer cells cope with increased energy demands (Miller et al., 2012). Therefore, for example, it would be interesting to determine how CE depletion influenced HeLa cell glycolysis rates.

Notably, CE depletion in HeLa cells caused morphological changes (Figure 5.6 C and D). Cells primarily became elongated and refractile, with a smaller population of cells becoming hypertrophic, flat and multinucleated. Both of these phenotypes are hallmarks of senescence. It would therefore be interesting to determine if CE depletion causes reduced cell proliferation over a longer time-course, and to investigate senescence markers such as β-galactosidase activity. C-MYC knockdown also caused morphological changes associated with senescence, although there was a greater proportion of flat hypertrophic cells (Figure 5.6 E and F). This phenotype has also been observed in other cell types upon C-MYC depletion (Florea et al., 2013; Zhuang et al., 2008) and tumour regression via C-MYC inhibition is dependent on senescence in certain mouse models (Wu et al., 2007). Additionally, CE and C-MYC knockdowns caused an apparent decrease in the formation of cell protrusions (Figure 5.6 C-F). Therefore, it could be investigated if CE knockdown reduced cell migration or invasion of HeLa cells. There are C-MYC target genes involved in senescence and cell motility (Gartel et al., 2001a; Staller et al., 2001b; Wang et al., 2013b; Yan et al., 2009), thus it would be of interest to determine if CE knockdown alters the expression of these genes. Indeed, in this chapter it was already shown that CE depletion in IMEC/C-MYC caused upregulation of p21

and p27 (Figures 5.3 A and B), which are each involved in distinct senescence pathways (Flores et al., 2014).

5.2.6 Comparing CE depletion and CDK7 inhibition

THZ1 is a specific inhibitor of CDK7, the kinase responsible for phosphorylating RNA pol II CTD at position 5. THZ1 covalently binds to a cysteine residue outside of the kinase domain, allosterically causing irreversible deactivation (Kwiatkowski et al., 2014). In vitro, this compound greatly perturbs co-transcriptional mRNA capping and causes RNA pol II pausing defects (Nilson et al., 2015). Furthermore, THZ1 treatment induces apoptosis in many cancer cell lines and in mouse models of triple negative breast cancer, neuroblastoma and small cell lung cancer driven by *MYC* family members (Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Nilson et al., 2015). Taking into account observations in this thesis, it is possible that mRNA capping inhibition contributes to THZ1 efficacy. It was investigated whether THZ1 treatment caused a similar cellular effect to CE inhibition. A fluorometric assay was used which measures the metabolic activity of cells, which be used as an approximation of cell viability. Since the cell lines used here proliferate at different rates, conditions were optimised (Figure 5.7). In previous experiments, cells were counted 72 hours post-*CE* siRNA transfection. However, a reduction in CE protein levels is not observed until 36-48 hours post-transfection. This means that cells were counted 24-36 hours after CE was depleted. THZ1 abolishes cellular S5p RNA pol II rapidly; after ~2 hours (Kwiatkowski et al., 2014), therefore fluorescence was measured 24 hours post-THZ1 treatment to compare CDK7 inhibition with CE depletion. HeLa cells, which were not sensitive to CE depletion, were somewhat sensitive to THZ1

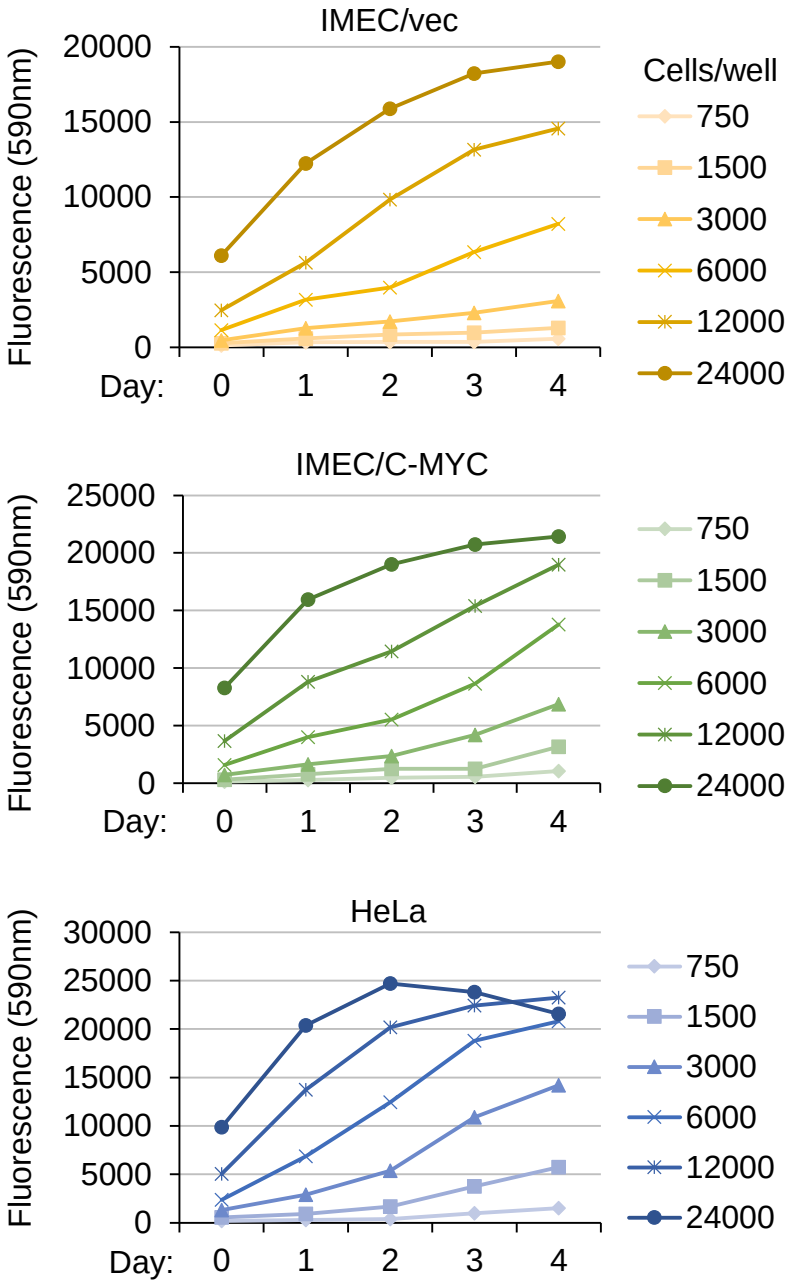

Figure 5.7: Optimising 96 well viability assays. IMEC/vec, IMEC/C-MYC and HeLa cells were seeded in 96 well plates with the indicated numbers of cells in each well. CellTiter Blue dye (containing resazurin) was added 0-4 days after plating. Metabolically active cells catalyse resazurin to resorufin which emits fluorescence at 590nm, therefore fluorescence is an approximation of cell viability. Individual experiment.

(Figure 5.8 A) with an IC_{50} of ~390nM, similar to HeLa cells treated with THZ1 for 72 hours in a previous study (IC_{50} 357nM) (Kwiatkowski et al., 2014). It should be noted that HeLa cells only ranked 671-most sensitive to THZ1 out of 1153 cell lines tested. In contrast, both IMEC/vec and IMEC/C-MYC were relatively insensitive to THZ1 when cultured with or without FBS, with the highest concentrations only causing a 40-50% decrease in cell viability (Figures 5.8 A and B). Therefore, C-MYC sensitises IMECs to CE depletion but not to CDK7 inhibition. Perhaps more drastic changes in IMEC viability would be observed after a longer treatment with THZ1. To determine if C-MYC knockdown desensitised HeLa cells to CDK7 inhibition, cells were transfected with *C-MYC* siRNA and treated with THZ1 after 72 hours. Preliminary results indicate that C-MYC knockdown does not drastically alter HeLa cell sensitivity to CDK7 inhibition (Figure 5.8 C). Therefore, HeLa cells are not sensitive to CE or C-MYC inhibition, but are sensitive to CDK7 inhibition in a C-MYC-independent manner.

IMECs were sensitive to CE depletion - but not CDK7 inhibition – in a C-MYC-dependent manner. Therefore, it was investigated if CE knockdown and THZ1 differentially affected expression of C-MYC and the C-MYC target gene *NCL*. S5p RNA pol II, S2p RNA pol II and endogenous C-MYC levels were reduced by THZ1 treatment in a dose-dependent manner (Figure 5.9), consistent with previous studies (Christensen et al., 2014; Kwiatkowski et al., 2014). NCL expression was elevated in IMEC/C-MYC cells, but was not depleted by THZ1 treatment (Figure 5.9). Reproducibly, the expression of exogenous C-MYC in IMEC/C-MYC was diminished with 75nM THZ1 but not with 375nM THZ1 (Figure 5.9). One potential explanation of the latter is that

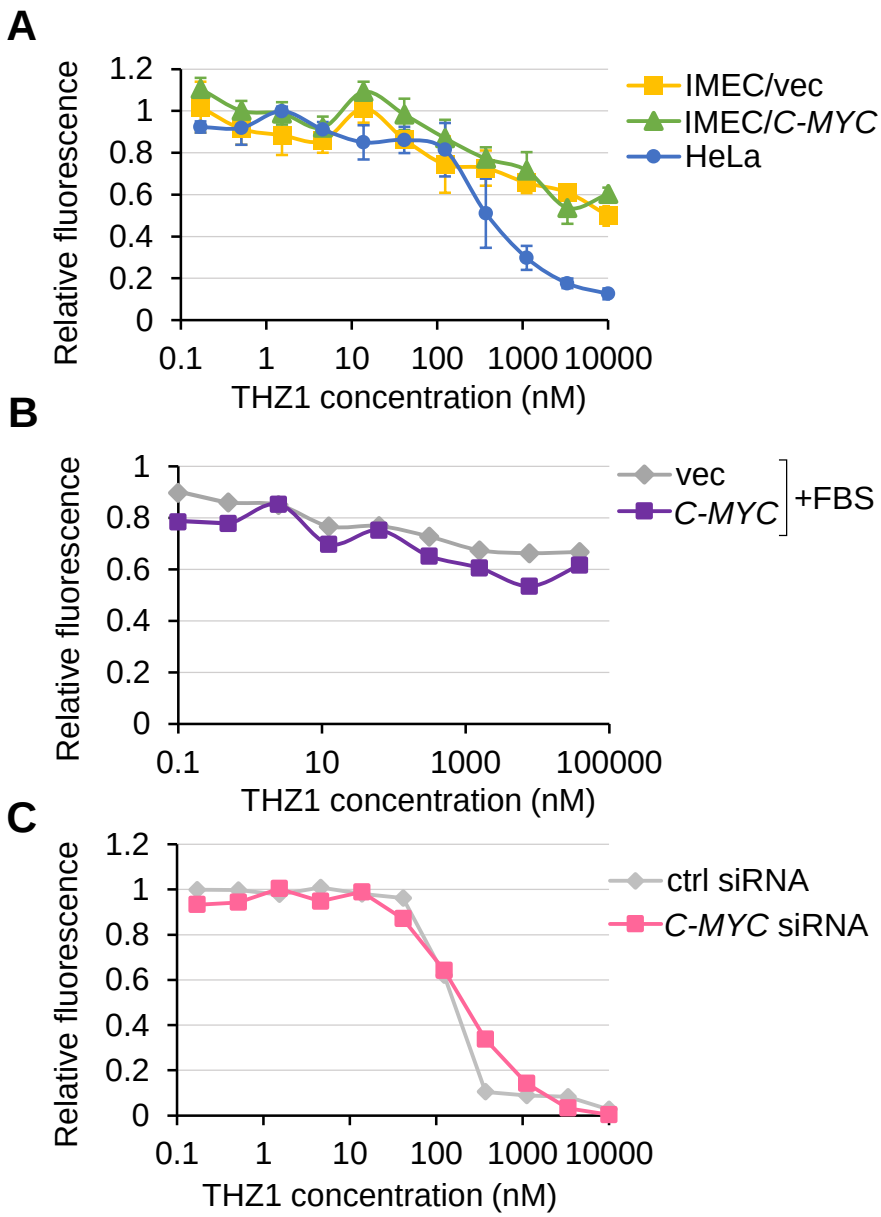

Figure 5.8: Modulating C-MYC in IMECs and HeLa cells has no effect on sensitivity to THZ1. (A) IMEC/vec, IMEC/C-MYC and HeLa cells were treated with the indicated concentrations of THZ1 or DMSO for 24 hours before cell viability was measured using Cell Titer Blue. 9000 cells seeded per well. Error bars represent standard error of the mean, n=3. (B) IMEC/vec and IMEC/C-MYC maintained in FBS were treated with the indicated concentrations of THZ1 or DMSO for 24 hours before cell viability was measured as above. 6000 cells seeded per well. Average of two independent experiments. (C) HeLa cells were transfected with 25nM *C-MYC* siRNA or non targeting control (ctrl). 5300 cells seeded per well. After 72 hours, cells were treated with the indicated concentrations of THZ1 or DMSO for 24 hours before cell viability was measured as above. Individual experiment.

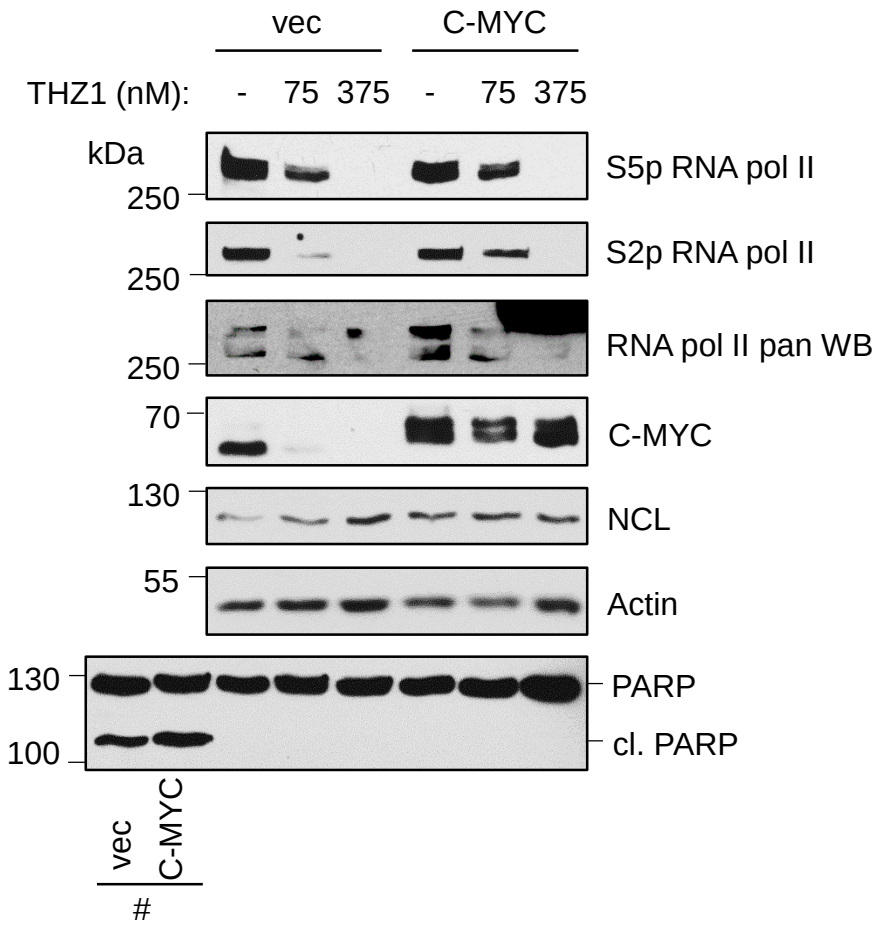

Figure 5.9: Investigating IMEC response to THZ1. IMEC/vec and IMEC/C-MYC were treated with DMSO (-) or the indicated concentrations of THZ1 for 24 hours before protein was extracted and analysed by Western blotting. $3x10^5$ cells were seeded per 6cm plate. # IMECs treated with 10μM MG132 for 24 hours as a positive control for apoptosis. Representative of two independent experiments.

RNA pol II is titrated away from endogenous promoters when global transcription is inhibited at higher THZ1 concentrations, resulting in more RNA pol II availability. Additionally, THZ1 treatment did not induce PARP cleavage (Figure 5.9), suggesting that IMECs were not undergoing apoptosis. Taken together, CDK7 inhibition and CE inhibition have distinct effects in IMECs since THZ1 does not display specificity towards cells with deregulated C-MYC.

5.3 Discussion

5.3.1 Summary

In this chapter it was shown that C-MYC-driven cells were sensitive to CE depletion, but not normal cells with basal C-MYC levels nor cells which are not driven by C-MYC. IMECs with deregulated C-MYC expression – and not normal IMECs – exhibited upregulation of negative regulators of the cell cycle p21 and p27 in response to CE depletion, consistent with CE being required for C-MYC-driven cell cycle progression. Importantly, CE depletion caused a significant reduction in anchorage-independent cell growth (a characteristic of malignant transformation) in IMECs with deregulated C-MYC, and breast cancer patients whose tumours express higher levels of *CE* have an unfavourable prognosis. Additionally, CE inhibition had distinct cellular effects to CDK7 inhibition, and could have more specificity towards the C-MYC response. Collectively, these data strongly suggest that targeting mRNA capping should be investigated as a potential strategy to selectively intervene with C-MYC-driven cancers.

5.3.2 C-MYC deregulation sensitises cells to CE inhibition

The finding that deregulated C-MYC induced CE dependency in IMECs is consistent with the differential dependency of C-MYC target genes on CE. Proliferation and anchorage-independent cell growth are indicative of cell fitness and the transforming capability of cells, which are controlled by a plethora of genes not necessarily regulated by C-MYC. Therefore, since there was a correlation between C-MYC target gene expression and IMEC transformation capability, CE inhibition may preferentially inhibit C-MYC target genes. Although

the effect of CE depletion in HeLa cells was not as drastic, changes in C-MYC target gene expression may confer the observed changes in cell morphology. Since C-MYC is deregulated in HeLa cells but C-MYC inhibition had no effect on HeLa cell number, it is possible that C-MYC was important for initial tumour development, but with the occurrence of further mutations was no longer required. In which case, perhaps sensitivity to CE inhibition is selective for C-MYC-dependent cancer cells. Alternatively, as previously suggested, proliferation changes may be masked by concomitant C-MYC-induced proliferation and apoptosis.

It is highly likely that cellular CE dependency varies depending on biological context. Ideally, CE knockdown should be performed in a panel of cancer cell lines with differential dependencies on C-MYC to comprehensively investigate if there is a correlation between C-MYC and CE dependency. Moreover, the dogma is that an oncogene and tumour-suppressor gene both be deregulated in order for oncogenesis to occur. Therefore, it would be interesting to determine if deactivation of a tumour suppressor gene (e.g. an anti-apoptotic gene) augments the sensitivity of IMECs to CE inhibition.

It is worth noting that soft agar transformation assays ascertain the ability of cells to form anchorage-independent colonies when CE is already depleted. Therefore, in this experimental arrangement, the importance of CE in early malignant transformation is tested. To test whether CE could be a valid molecular target for cancer therapies, ideally CE should be withdrawn after colony formation to mimic treating a tumour. It is unlikely that siRNA would be able to transfect colonies suspended in soft agar, therefore perhaps using cells expressing an inducible *CE* shRNA construct and inducing expression post-

colony formation could determine whether CE inhibition causes colony regression.

5.3.3 Potential involvement of CE in specific breast cancers

CE transcript levels in basal-like breast cancer/TNBC tumours inversely correlated with patient survival. If high CE expression has a role in sustaining basal-like/TNBC tumours, this is of particular interest since these cancers tend to be unresponsive to available therapies, demonstrating an unmet need for new molecular targets. Curiously, *C-MYC* is also often deregulated in basal-like/TNBCs, and a *C-MYC* target gene signature is apparent in basal/TNBC subtypes but not others (Alles et al., 2009; Chandriani et al., 2009; Gatza et al., 2010; Horiuchi et al., 2012). Spliceosome inhibition in C-MYC-dependent pre-clinical models of TNBC was shown to diminish tumourigenesis and metastasis (Hsu et al., 2015), highlighting that these cancers are particularly dependent on mRNA processing. Basal-like breast cancers/TNBCs are often associated with perturbation of the breast cancer 1 (*BRCA1*) tumour suppressor pathway involved in DNA repair, transcription, cell cycle regulation and apoptosis (Deng, 2006; Lakhani et al., 2002). Not surprisingly, there are reports of *BRCA1* and *C-MYC* co-deregulation in these contexts (Adem et al., 2004; Grushko et al., 2004). In fact, their protein products physically associate with each other and BRCA1 negatively modulates C-MYC transcriptional activity (Li et al., 2002; Wang et al., 1998). Furthermore, BRCA1 perturbs CDK7 activity and thus downmodulates RNA pol II CTD S5 phosphorylation (Moisan et al., 2004), therefore BRCA1 has the potential to control CE recruitment. It would therefore be interesting to determine if disruption of the *BRCA1* pathway increases the sensitivity of cells to CE depletion; with or without C-MYC deregulation.

Moreover, the effect of CE depletion could be compared in cancer cell lines with mutant or WT *BRCA1*.

Additionally, *CE* transcript levels in mesenchymal breast tumours was anti-correlative with patient survival. C-MYC is known to promote EMT in mammary epithelial cells (Cho et al., 2010; Cowling and Cole, 2007a) and p21 elevation has been shown to reverse C-MYC-induced EMT (Liu et al., 2009). It would thus be interesting to analyse if CE knockdown (via p21 elevation or otherwise) represses C-MYC-mediated EMT.

It should be noted that overexpressing CE in the cells analysed here had no effect on proliferation (data not shown) suggesting that it is not a classical oncoprotein. However, cells may develop a dependency on CE upon transformation, which could provide a survival advantage to tumour cells which happen to have elevated levels of CE. This phenomenon is termed 'non-oncogene addiction' (Nagel et al., 2016).

5.3.4 CE inhibition and CDK7 inhibition elicit different cellular responses

IMECs exhibited a C-MYC-dependent response to CE depletion, but not THZ1-mediated CDK7 inhibition. Although THZ1 was shown to decrease mRNA capping in vitro (Nilson et al., 2015), the distinct cellular response to CE depletion indicates that this is not THZ1's primary mode of action in these cells. There are additional effects of THZ1 treatment in cells such as S5p and S2p RNA pol II depletion (not consistently observed in IMECs upon CE knockdown), S7p RNA pol II depletion and RNA pol II pausing defects (Kwiatkowski et al.,

2014; Nilson et al., 2015). This likely results in additional transcriptional events being perturbed and causing a different cellular response than CE knockdown. Deregulation of MYC family members was thought to confer sensitivity of neuroblastoma, small cell lung cancer and triple-negative breast cancer cells and mouse models to CDK7 inhibition in previous studies, which is somewhat contrary to cell lines used in this study. However, this was proposed to be due to *MYC* genes being regulated by super-enhancers: *cis*-acting genomic regions with hubs of transcription machinery to promote expression of particular genes, rendering *MYC* and other super-enhancer associated oncogenes particularly sensitive to CDK7 inhibition. Since these genes acquire super-enhancer regulation during malignant transformation, cellular 'addiction' to super-enhancer-driven transcription may also require additional oncogenic events such as deregulation of other transcription factors or chromosomal rearrangements. Additionally, it should be noted that exogenous C-MYC in IMEC/C-MYC is not expressed from its endogenous location in genome and therefore is not regulated by its endogenous regulatory regions. This might explain why deregulated C-MYC expression in IMEC/C-MYC is less robustly perturbed by THZ1 compared to endogenous C-MYC in IMEC/vec. Nonetheless, these results are consistent with CE depletion and THZ1 acting via distinct mechanisms. For proper comparison of CE depletion and CDK7 inhibition efficacy in C-MYC-driven transformation, C-MYC-dependent cell lines should be used which express endogenously deregulated C-MYC.

Chapter 6 : Final discussion and future work

6.1 Summary

C-MYC is a transcription factor and a driver of diverse cancer types. Amongst its many functions, C-MYC upregulates formation of the mRNA cap, which is important for transcript stability, processing and translation. mRNA capping is catalysed by two enzymes: CE which catalyses addition of the 5' inverted guanosine moiety; and RNMT-RAM which methylates the guanosine group. Previous studies have focussed on the role of RNMT-RAM in mRNA capping regulation by C-MYC. Therefore, in this thesis the involvement of CE – the enzyme which initiates cap synthesis – was investigated. It was confirmed that CE interacted with the RNA pol II CTD phosphorylated at S5 residues and that C-MYC increased this phosphorylation event in cells. C-MYC regulated CE recruitment to S5p RNA pol II and SPT5 complexes; likely in a CDK7-dependent manner. Moreover, on chromatin, C-MYC mediated CE recruitment to its target genes. The expression of C-MYC target genes, and also C-MYC itself, were highly dependent on CE. However, upon CE depletion, the change in C-MYC target genes exceeded the change in C-MYC expression, suggesting these two modules were partially uncoupled. This highlights a potential feed-forward loop (Figure 6.1). A role for CE in the regulation of RNA pol II transcription was also identified, although whether this impacts C-MYC function is not yet clear. Surprisingly, in cells with basal C-MYC levels or cells insensitive to C-MYC, the vast majority of cellular CE was superfluous for cell viability. However, CE was required for in vitro neoplastic growth driven by deregulated C-MYC. CE depletion upregulated negative regulators of the cell cycle selectively in the presence of deregulated C-MYC, consistent with CE being

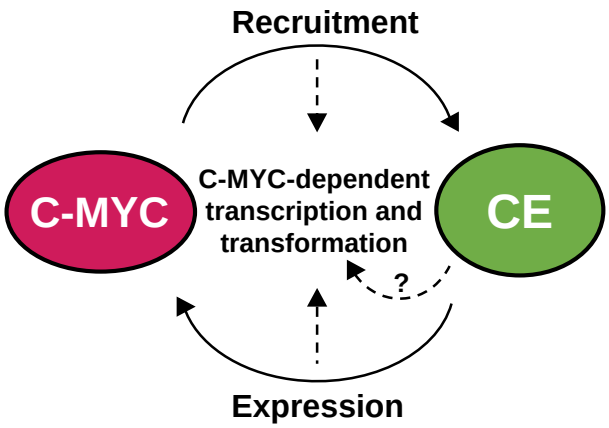

Figure 6.1: The interplay between C-MYC and CE. A bidirectional relationship exists between C-MYC and CE, in which C-MYC regulates CE recruitment and CE regulates C-MYC expression. Together, CE and C-MYC regulate C-MYC target genes and C-MYC-driven transformation. It is also possible that CE regulates C-MYC target genes independently of C-MYC.

required for C-MYC-driven cell cycle transit. Upon comparison with CDK7 inhibition (which inhibits both transcription and mRNA capping), CE depletion presented a distinct cellular response and exhibited more specificity towards cells with deregulated C-MYC. Finally, in specific subtypes of breast cancer which commonly carry deregulated C-MYC, *CE* expression correlated with poor prognosis. Taken together, a direct mechanism by which C-MYC mediates mRNA cap formation was discovered, illustrating that C-MYC upregulates mRNA capping by synchronising multiple activities. Moreover, CE was identified as a potential therapeutic target to selectively intervene with C-MYC-driven cancers, and this warrants further investigation.

6.2 Discussion

6.2.1 CE regulation in C-MYC-dependent gene expression

Studies in this thesis have revealed that C-MYC regulates CE, demonstrating that C-MYC synergises both mRNA guanylylation and cap guanosine methylation to drive mRNA capping via several mechanisms (Figure 6.2). In summary, C-MYC increases the expression of CAK module components within TFIIH (including CDK7) and increases its recruitment to transcription start sites (Cowling and Cole, 2007b; Posternak et al., 2017). This promotes RNA pol II CTD S5 phosphorylation, which directly recruits CE and (through an uncharacterised mechanism) also recruits RNMT-RAM (Aregger and Cowling, 2013; Cowling and Cole, 2007b; Ho et al., 1998; Lombardi et al., 2016; Posternak et al., 2017). Binding of S5p RNA pol II to CE stimulates CE activity, and C-MYC indirectly promotes RNMT-RAM activity via regulating SAHH expression (Fernandez-Sanchez et al., 2009; Ho and Shuman, 1999; Lombardi et al., 2016; Moteki and Price, 2002). From a wider perspective, this contributes to the multiple mechanisms by which C-MYC promotes gene expression. C-MYC increases the transcription of thousands of genes (Dang, 2014; Littlewood et al., 2012), and simultaneous upregulation of transcript capping will stabilise transcripts during transcription; increasing the likelihood that they will be processed properly, exported into the cytoplasm and translated. Concurrently, C-MYC transcriptionally upregulates components of the eIF4F complex (including the cap-binding protein eIF4E) (Lin et al., 2008; Lin et al., 2012a) thereby enhancing translation via recruiting capped mRNA molecules to the ribosome (Figure 6.2). C-MYC also enhances translation via increasing tRNA synthesis and ribosome biogenesis by various mechanisms

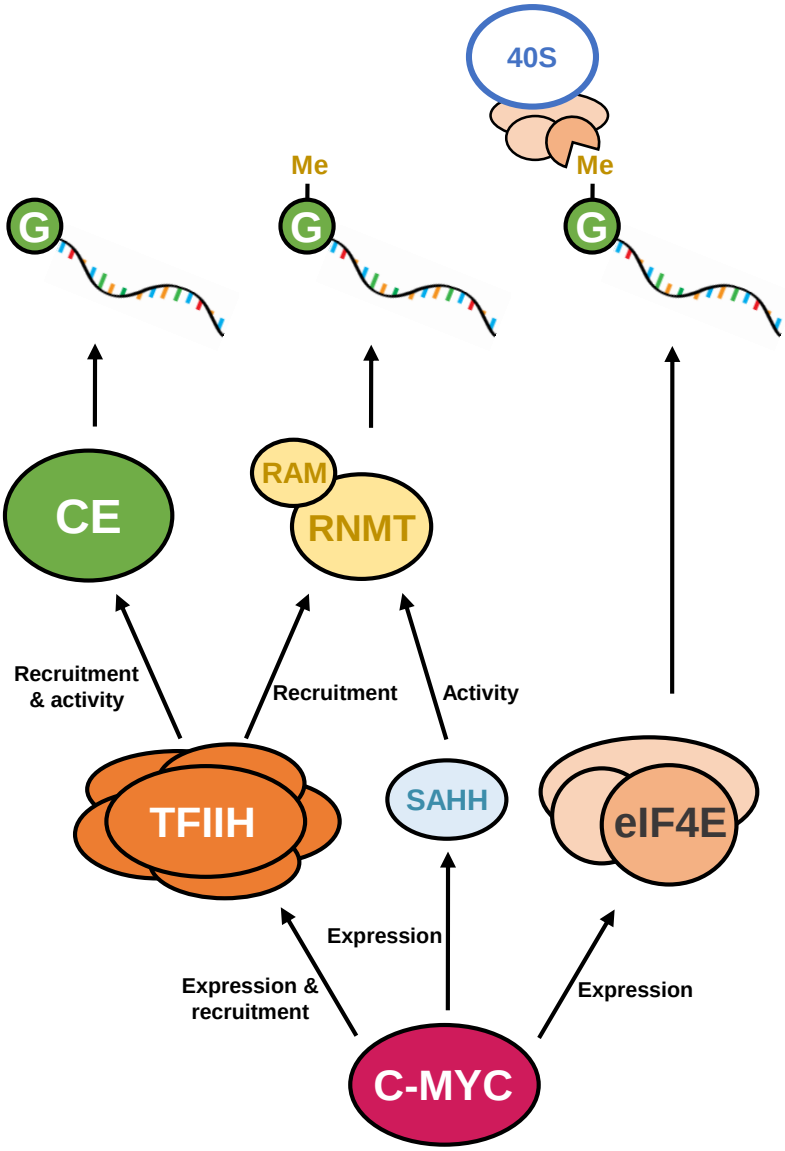

Figure 6.2: C-MYC co-ordinates several aspects of cap-dependent translation. C-MYC promotes the expression and recruitment of TFIIH subunits, which via the CDK7 kinase module regulates the recruitment of CE and RNMT to transcription start sites (Cowling and Cole, 2007b; Posternak et al., 2017). CDK7-dependent phosphorylation of the RNA pol II CTD also stimulates CE activity (Ho and Shuman, 1999; Moteki and Price, 2002). C-MYC upregulates SAHH, which promotes RNMT activity (Fernandez-Sanchez et al., 2009). Additionally, C-MYC induces expression of eIF4F complex components including eIF4E, enhancing cap-dependent translation (Lin et al., 2008; Lin et al., 2012a). C-MYC also promotes translation in general by modulating tRNA production and ribosome biogenesis (not shown, van Riggelen et al., 2010b). 40S, small ribosomal subunit.

(Gomez-Roman et al., 2006; Ji et al., 2011; van Riggelen et al., 2010b). The model emerges that C-MYC promotes concomitant transcription, mRNA capping and translation of target genes to encourage rapid transduction of genetic information and protein synthesis. The relative importance of each node in C-MYC function is not clear, but this could vary in a cell- and context-dependant manner to govern differential gene expression.

6.2.2 CE specificity

The global specificity of CE in human cells has not yet been investigated. Based on studies in this thesis, it can be hypothesised that CE inhibition has a biased effect on C-MYC-induced genes and thus reverses the effect of C-MYC deregulation without causing adverse cellular effects. Observations alluding to this include the negative correlation between the effect of C-MYC overexpression and CE depletion on C-MYC target genes. Of note, C-MYC-repressed genes are either unaffected or upregulated in response to CE depletion, arguing against CE activity being non-specific. Moreover, CE depletion reverses indicators of cell transformation in the presence of C-MYC deregulation to levels comparable to that in cells without C-MYC deregulation. It is worth noting that E2F1 – and probably other transcription factors – enhance CDK7 activity (Aregger and Cowling, 2012). Therefore, other transcription factors might depend on CE to drive their transcriptional programmes. In previous studies, partial inhibition of CDK7 did not impact gene expression globally but inhibited capping and expression of specific transcripts (Kanin et al., 2007; Kwiatkowski et al., 2014; Viladevall et al., 2009), illustrating that CDK7-dependent capping has the potential to be regulated in a gene-specific manner. Taken together, CE specificity may arise from transcription factors such as C-

MYC driving CDK7-dependent CE recruitment. During basal conditions, these transcripts may not be capped; instead undergoing internal ribosome entry site (IRES)-dependent/cap-independent translation (Komar and Hatzoglou, 2011). Alternatively, the differential dependency of certain genes on CE may be mediated indirectly by heightened transcriptional or translational burden, such as that upon C-MYC deregulation (discussed below). Global gene expression analyses are required to better understand CE specificity.

6.2.3 Selective tolerance to CE depletion

As determined by genetic knockout, yeast capping enzymes are essential for viability (Mao et al., 1995; Schwer and Shuman, 1994; Shibagaki et al., 1992; Shuman et al., 1994; Tsukamoto et al., 1997; Wang and Shuman, 1997). Genome-wide knockout screens using the CRISPR system in seven human cell lines (including HeLa cells) across two different studies have also identified *CE* and *RNMT* as essential genes (Hart et al., 2015; Wang et al., 2014). The observation in this thesis that CE is somewhat dispensable in certain cells was therefore unexpected. There are many possible explanations for this observation. One likely interpretation, due to *CE* being an essential gene, is that CE is in excess and therefore not limiting in these cells. For example, in conditions with low basal levels of transcription, perhaps a small fraction of cellular CE suffices to maintain mRNA capping. In cells with deregulated C-MYC, it is likely that CE is required in abundance but is highly used and therefore limiting; posing a burden upon the cell. Since mRNA capping is important for splicing (Inoue et al., 1989; Lewis and Izaurflde, 1997), another factor contributing to tolerance of CE inhibition could be mRNA surveillance mechanisms removing any aberrantly spliced transcripts upon CE

depletion, thereby preventing synthesis of potentially toxic proteins (Egecioglu and Chanfreau, 2011; Kawashima et al., 2009). Global elevation of transcription could saturate these mechanisms. Consistently, in another study spliceosome inhibition is well tolerated in normal cells with basal levels of transcription, whereas it is not tolerated in the same cells upon C-MYC activation (Hsu et al., 2015). There are many other cellular stresses emerging from C-MYC deregulation which could indirectly heighten cell sensitivity to CE depletion (Nagel et al., 2016).

Alternatively, perhaps cells can compensate in ways which we do not yet understand. For example, IRES-dependent translation might compensate for lack of cap-dependent translation in certain situations, and this could require factors which are limiting in cells with deregulated C-MYC. Indeed, previous studies have reported a switch from cap-dependent to cap-independent translation in conditions of cellular stress (Braunstein et al., 2007; Komar and Hatzoglou, 2005). Interestingly, C-MYC overexpression in mouse B lymphocytes increases global cap-dependent translation while preventing the switch to cap-independent translation in mitosis, which is crucial for translation of specific IRES-containing mRNAs important in mitotic progression (Barna et al., 2008). This would be consistent with C-MYC-overexpressing cells being deficient in IRES-dependent translation and therefore more dependent on mRNA capping and cap-dependent translation. Further investigation is required to dissect the exact mechanisms governing cellular tolerance and sensitivity to CE inhibition.

6.2.4 CE as a potential therapeutic target

A therapeutic agent must display a sufficiently large therapeutic window. Identification of synthetic lethal genetic interactions with oncogenes provides a rational route to identify such therapies (Cermelli et al., 2014; Nijman, 2011). Synthetic lethality occurs when perturbation of two genes causes loss of cell fitness, whereas one perturbation is tolerated by the cell. These studies indicate that CE inhibition is synthetic lethal (or synthetic sick) with deregulated C-MYC (Figure 6.3). These observations are complementary to the finding that inhibition of cap methylation is synthetic lethal with C-MYC deregulation (Fernandez-Sanchez et al., 2009). Moreover, spliceosome inhibition was previously shown to be synthetic lethal with C-MYC activation (Hsu et al., 2015). It thus emerges that several steps of co-transcriptional mRNA processing are specifically important for C-MYC-dependent transformation, which is consistent with C-MYC widely increasing cellular transcriptional and translational load. Interestingly, in another study CE inhibition was shown to be synthetic lethal with tuberous sclerosis 1 (*TSC1*) or *TSC2* deletion (Housden et al., 2015). Defective TSC1/2 regulation is associated with various cancers (Mieulet and Lamb, 2010). TSC1/2 are upstream negative regulators of mTOR complex 1 (mTORC1) which activates eIF4E and thus cap-dependent translation (Showkat et al., 2014). There are reports of C-MYC functionally antagonising and transcriptionally downregulating TSC2 (Li et al., 2003b; Ravitz et al., 2007; Rosner et al., 2003; Tapon et al., 2001), suggesting that C-MYC and TSC1/2 exist within the same pathway. Since C-MYC and TSC1/2 both regulate eIF4E (positively and negatively, respectively), perhaps aberrant control of the eIF4E – cap-dependent translation axis sensitises cells to CE inhibition (Figure 6.3). This is reminiscent of the finding that inhibition of the eIF4F complex is synthetic

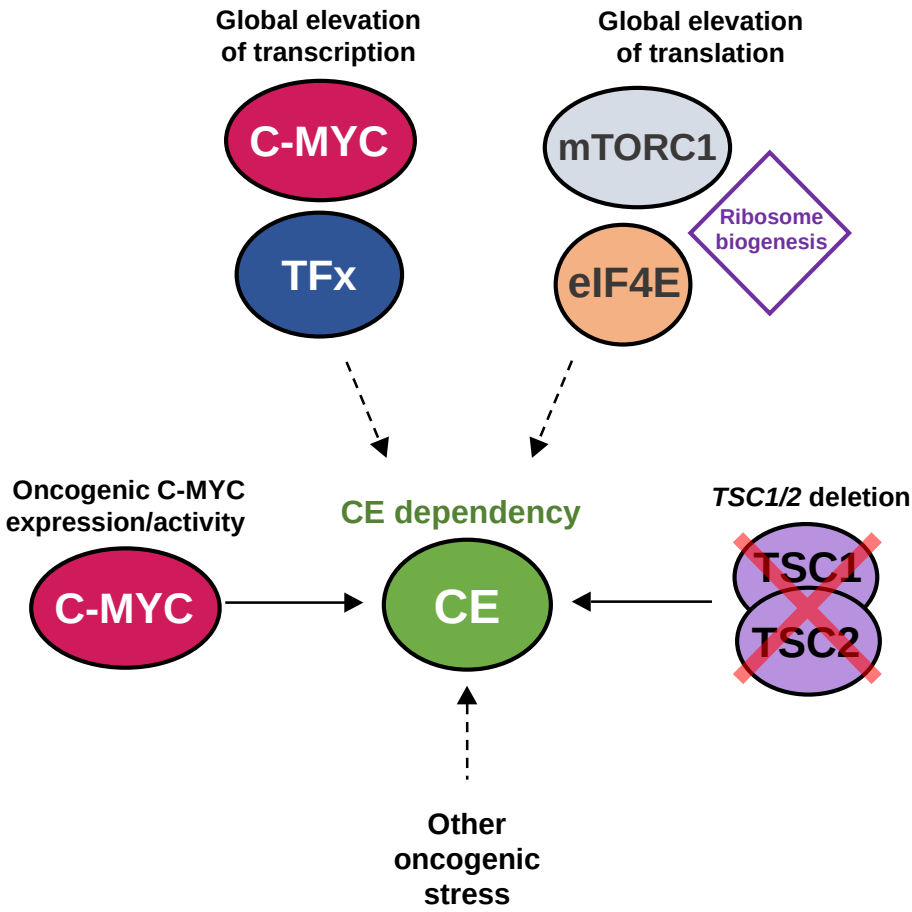

Figure 6.3: Development of CE dependence. Known and potential mechanisms by which cells develop CE dependence are shown (denoted by solid and dashed arrows, respectively). In this thesis, C-MYC deregulation was shown to confer cellular dependence on CE. In another study, *TSC1* or *TSC2* deletion induced CE dependency (Housden et al., 2015). Potential factors contributing to CE dependence include global elevation of transcription (e.g. by C-MYC or other transcription factors; TFx), global elevation of translation (e.g. via C-MYC upregulating eIF4E and ribosome biogenesis), or activation of other stress pathways specific to certain transformed cells (Nagel et al., 2016).

lethal with C-MYC dysregulation (Lin et al., 2012a). In such a scenario, cells may be addicted to eIF4E cap-dependent translation and inhibition of CE could thus curb eIF4E-dependent protein synthesis.

The emergence of personalised medicine in cancer treatment, in which a therapy is chosen based on the tumour genome/transcriptome, has had some positive impact but tumour relapse is a problem (Ashley, 2016). Cancer is a heterogeneous cellular disease, therefore targeting a specific gene to attain complete and sustained tumour submission is challenging. Increased transcription and translation are common features of cancer cells (Bhagwat and Vakoc, 2015; Bhat et al., 2015; Bywater et al., 2013). Targeting a process like mRNA capping – which has the potential to interfere with both of these processes – could thus exploit the transcriptional/translational burden on cancer cells irrespective of their oncogenic aberrations (Figure 6.3). Oncogene transcripts tend to be intrinsically unstable compared to the average human mRNA, which has a half-life of 10 hours (Wilusz et al., 2001; Yang et al., 2003). For example, transcripts of *C-MYC*, *C-FOS* and *C-JUN* oncogenes all have half-lives of 10-30 minutes (Bertani et al., 1989; Dani et al., 1984; Shyu et al., 1989). Therefore, uncapped oncogenic mRNAs might be more susceptible to degradation upon CE inhibition. Furthermore, a mechanism by which oncogenes (including *C-MYC*) can become deregulated is via stabilisation of their transcripts (Khabar, 2017; Weidensdorfer et al., 2009), which has the potential to be counteracted by CE inhibition. Another issue arising from the use of targeted cancer therapies is that cells can adapt in response to drug treatments via mutation of the target protein (Zahreddine and Borden, 2013). Therefore, targeting an essential gene like *CE*, which is not prone to mutation in

tumour cells (Forbes et al., 2017) is a favourable strategy. Globally defining the responsiveness of the transcriptome to CE inhibition in transformed and normal cells would help elucidate the mechanisms of CE dependency and indicate whether targeting CE/mRNA capping is a viable anti-cancer strategy.

Prior to these studies, CE regulation has received minimal attention. On the other hand, there have been some investigations of RNMT-RAM in the context of cancer cells, and these studies provide further rationale for targeting mRNA capping as a therapeutic strategy. As previously discussed, RNMT-RAM is regulated by C-MYC and E2F1 oncogenic transcription factors (Cole and Cowling, 2009b; Dunn and Cowling, 2015). Furthermore, ectopic RNMT overexpression induces cell transformation; on its own but also in collaboration with *C-MYC* and *RAS* oncogenes (Cowling, 2009). RNMT-RAM also regulates the expression of *CCND1* and *C-MYC* oncogenes (Cowling, 2009; Dunn et al., 2016). Although the global impact of RNMT-RAM on cancer cell gene expression is not yet known, analyses in ESCs highlighted that RAM influences adhesion-associated genes (Grasso et al., 2016). If the same is true in cancer cells, this might implicate RNMT-RAM in the control of cancer cell invasion. Moreover, since RAM is required for stem cell pluripotency (Grasso et al., 2016), it may be also be important in cancer stem cells (Kreso and Dick, 2014). More comprehensive studies are required to identify the best molecular target for mRNA capping inhibition.

6.3 Future work

In this thesis, CE was identified as a novel mediator of C-MYC-dependent transformation. However, this research raises many questions which require exploration.

6.3.1 How does CE influence mRNA metabolism?

There is evidence for the mRNA cap promoting transcription, mRNA stability, splicing, 3' end processing, nuclear export and translation (Lewis and Izaurflde, 1997; Ramanathan et al., 2016). However, the relative contribution of CE to these processes in human cells is not clear. In this thesis it was shown that CE influences transcript levels of C-MYC and C-MYC target genes, consistent with a role for CE in transcription, mRNA stability or transcript processing. Conversely, other studies show that RNMT-RAM influences C-MYC target gene transcript polysome loading and protein synthesis rather than altering mRNA levels, which is indicative of translational regulation (Cowling, 2009; Cowling and Cole, 2007b; Posternak et al., 2017). In light of findings that both CE and RNMT-RAM are key for oncogenic C-MYC function, it will be important to fully characterise and compare their functions in mRNA metabolism. This should be tested in isogenic non-transformed cells and cells carrying deregulated C-MYC by knocking down CE and RNMT-RAM in parallel. They could also be inhibited in combination to determine whether this has a synergistic effect, and this would shed light on whether they differentially regulate gene expression. Extended RNA pol II ChIP-seq and GRO-seq (nascent pre-mRNA labelling with bromouridine) would highlight whether CE or RNMT-RAM impacts RNA pol II processivity and mRNA synthesis. Using a

transcription inhibitor and tracking mRNA decay over a time-course would indicate whether the capping enzymes impact transcript stability. Moreover, the relative levels of nascent and mature transcripts, and prevalence of poly(A)-tailed transcripts, could be measured to detect any processing impairments. Polysome profiling – in which the relative abundance of transcripts with actively translating ribosomes is measured – could be performed to determine how CE alters translation relative to RNMT-RAM. Alternatively, ribosome profiling (Ribo-seq) could be performed to globally analyse ribosome-protected fragments on mRNA molecules and thus determine the relative contribution of CE and RNMT-RAM to translation regulation. These experiments would not only yield a greater understanding of CE and RNMT-RAM function, but would also provide insight into the mechanisms and consequences of C-MYC-mediated mRNA capping. Furthermore, studies here and elsewhere have highlighted a potential role of CE in RNA pol II pause release, independent of its capping activity. Therefore, the guanylyltransferase-dead mutant of CE (K294A) could be expressed in cells upon knockdown of endogenous CE before analysing C-MYC target gene expression. This would clarify the relative contribution of CE recruitment and mRNA capping to C-MYC-driven RNA pol II transcription.

6.3.2 Does CE exhibit transcript specificity?

In this thesis it was shown that C-MYC induces CE recruitment to transcription complexes. Moreover, C-MYC target gene transcripts are particularly sensitive to CE knockdown in conditions when C-MYC is deregulated. This is consistent with C-MYC recruiting CE to drive expression of its target genes. To determine if CE is truly used as a co-factor in C-MYC-driven transcription, a fusion construct could be made linking the *C-MYC* DBD (which

alone is insufficient for C-MYC function) to *CE*, and the ability of the C-MYC DBD-CE fusion protein to activate transcription tested. However, how CE regulates the rest of the genome is not known. Performing RNA sequencing (RNA-seq) and proteomics would help determine if C-MYC target genes or indeed other sets of genes are particularly responsive to CE knockdown. Moreover, CE ChIP-seq could be performed to help differentiate which genes are directly regulated by CE from secondary effects. This could be performed in parallel with C-MYC ChIP and RNA pol II ChIP to determine if CE is preferentially recruited to C-MYC target genes or highly transcribed genes. There are likely additional features which influence the sensitivity of transcripts to CE inhibition, such as mRNA instability elements and 5' UTR structures, which should also be interrogated.

6.3.3 What are the mechanisms of CE dependency?

Studies here determined that C-MYC-driven cells are particularly dependent on CE, whereas elsewhere it was shown that cells carrying deletion of the tumour suppressor complex TSC1/TSC2 within the mTOR pathway also exhibit heightened dependency on CE (Housden et al., 2015). The mechanisms governing these vulnerabilities are not known. A common consequence of C-MYC and mTORC1 pathway deregulation is heightened cap-dependent translation, converging on eIF4F (Lin et al., 2008; Pourdehnad et al., 2013; Showkat et al., 2014). Therefore, the oncogenic, cap-binding eIF4E subunit of eIF4F could be overexpressed in cells to determine if this induces CE dependency. Moreover, a panel of cell lines with different basal protein synthesis rates could be tested to determine if sensitivity to CE inhibition correlates with translational output. It could also be investigated whether C-

MYC deregulation and *TSC1/TSC2* depletion synergise in conferring CE dependency to determine whether they act via the same mechanism.

It was shown that high expression of *CE* in basal-like breast cancer/TNBC predicts an unfavourable patient outcome. Since perturbation of the BRCA1 tumour suppressor pathway is a common feature of these tumours, the effect of BRCA1 depletion on CE dependency could be tested. C-MYC and BRCA1 both influence RNA pol II transcription (positively and negatively, respectively) (Dang, 2014; Mullan et al., 2006), therefore perhaps elevated mRNA synthesis is a governing factor of CE dependency. This could be tested by correlating nascent transcription rates in a cell line panel with cellular sensitivity to CE inhibition. Additionally, it could be tested whether a sub-lethal dose of a transcription inhibitor (for example the CDK7 inhibitor THZ1) desensitises cells to CE inhibition, to investigate whether basal transcription activity is a determining factor of CE dependency.

Consistent with *CE* being an essential gene, it is likely that CE dependency is determined by whether it is limiting under certain conditions. To confirm this, gel filtration could be performed to determine the relative abundance of cellular monomeric CE and CE in transcription complexes. Moreover, it could be tested whether capping still occurs in cells upon CE knockdown using a N7-methylguanosine antibody or recombinant eIF4E to capture capped transcripts, and whether the absence of the cap correlates with CE dependency. Finally, although *CE* knockout was described as lethal in seven human cell lines including HeLa cells (Hart et al., 2015; Wang et al., 2014), it would be interesting to attempt CRISPR-mediated knockout of *CE* in IMECs since CE siRNA-mediated knockdown has a seemingly minimal effects in these cells.

6.3.4 Is targeting mRNA capping a viable therapeutic strategy?

In this thesis it was shown that CE knockdown specifically interferes with functions of deregulated C-MYC, and leaves untransformed (or C-MYC-independent) cells largely unaffected. This presents strong rationale to consider CE as a potential therapeutic target in C-MYC-driven cancers, although a number of experiments would substantiate this hypothesis.

Firstly, the efficacy of CE knockdown should be tested over a larger range of non-transformed and cancer cell lines. Of particular interest would be those exhibiting and lacking C-MYC deregulation for comparison. *TSC1/TSC2* mutant cancer cell lines and basal-like/TNBC cell lines could also be included in analyses. If CE knockdown proves to perturb cancer cell growth, following CE depletion cells could be injected into nude mice to determine their ability to form tumours in vivo. Since RNMT and SAHH also mediate C-MYC-driven transformation, it might be favourable to analyse RNMT and SAHH depletion in parallel to CE depletion in order to determine the best molecular target for mRNA capping inhibition.

Studies here show that CE inhibition is well-tolerated by non-transformed cells, and thus may display a therapeutic window in vivo. However, since *CE* is an essential gene, it would be favourable to determine whether sustained or enhanced CE knockdown has a cytotoxic effect on non-transformed cells, and to what extent CE inhibition can be tolerated. Human cell lines and mouse models conditionally expressing *CE* short hairpin RNA (shRNA) could be made to determine the longer-term effects of CE inhibition in vitro and in vivo. The

latter could be crossed to mouse models of C-MYC-driven Burkitt's lymphoma or TNBC to determine if CE is required for the oncogenic potency of C-MYC.

A specific inhibitor of CE would be more therapeutically relevant than genetic inhibition. The immunosuppressive agent mizoribine monophosphate (MZP) has been identified to inhibit human CE via an allosteric mechanism which perturbs RNA 5' guanylylation (Picard-Jean et al., 2013). However, MZP is also a potent inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), which mediates guanine nucleotide biosynthesis. Moreover, MZP is not particularly effective at inhibiting CE in vitro (IC_{50} 80μM). Studies presented in this thesis warrant the development of a potent, specific CE inhibitor to truly determine whether pharmacological inhibition of CE is a potential therapeutic strategy.

Chapter 7 : Bibliography

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